University of Reading

Chemically modified hydroxyethyl cellulose as a new excipient for transmucosal delivery

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Declaration of Original Authorship

The work described in this thesis was performed in the Department of Pharmacy of the University of Reading, United Kingdom between September 2019 and September 2022. I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Fhataheya Buang

Date: 14/07/2023

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Abstract

In the pharmaceutical industry, excipients such as cellulose and its derivatives are often used in the formulation of dosage forms as binders for tablets, viscosity enhancers, gelling agents, coating agents, etc. In addition to its basic functionality, this polymer can be modified to provide additional properties that optimise its performance in a particular application. An example of properties that are of great interest is mucoadhesion. The mucoadhesive properties allow greater contact between the formulation and the oral mucosa for a slow release of the active ingredient. Among the cellulose derivatives, hydroxyethyl cellulose (HEC) has weak mucoadhesive properties. However, it can be improved by modification with unsaturated groups such as methacryloyl, maleimide, acryloyl and divinyl sulfone. The newly modified HEC can interact with mucin glycoproteins by forming covalent bonds between the electronegative unsaturated end groups of the modified polymer and the less electronegative parts of the mucin glycoprotein (cysteine) via the Michael addition reaction. In this thesis, we have synthesised HEC with methacryloyl, maleimide, acryloyl and divinyl sulfone groups. The successful synthesis of the new polymers was validated using ^{1}H NMR and FTIR. It was further quantified by either ^{1}H NMR, HPLC and/or elemental analysis. These modified polymers were developed into various blank dosage forms (wafers, films, spray-coated tablets and microparticles) to determine their mucoadhesiveness using a texture analyser. A safety study was performed using planarian acute toxicity assays and planarian fluorescent toxicity assays. The safety study was further supported by *in vitro* toxicity assay using Caco-2 cells. Our findings suggest that synthesis process of all HEC derivatives was successful and regardless of molar ratio, the modified HEC with methacryloyl, maleimide, acryloyl and sulfone groups has improved the mucoadhesiveness of the native HEC. Additionally, the newly modified excipients are water soluble, versatile in dosage form development and easy to synthesis (one-pot synthesis method) under normal environmental conditions. These results support the idea that non-ionic HEC modified with unsaturated groups such as methacryloyl, maleimide, acryloyl and sulfone groups significantly improves the mucoadhesive properties of native HEC. Therefore, modified HEC with methacryloyl, maleimide, acryloyl and sulfone groups has great potential as a new multifunctional excipient for transmucosal drug delivery, as it has the advantage of being mucoadhesive yet retaining the non-ionic nature of HEC derivatives. This may promote greater compatibility with charged drug molecules in dosage form formulations.

List of conferences and presentations

1. The International Conference and Exhibition on Pharmaceutical Sciences and Technology 2020 (PST2020), Bangkok, Thailand on 19-20 May 2020 – Oral
2. Pharmacy Virtual Ph.D. Conference 2020, University of Reading, UK on 2nd – 3rd July 2020 – Poster
3. Pharmacy Ph.D. Virtual Conference 2021, University of Reading, UK on 14th – 15th April 2021 – Oral
4. Formulating for Adhesion: Sticking with it!, RCS Formulation and Science Technology Interest Group on 22 September 2021 – Oral
5. Pharmacy PhD Showcase 2022, University of Reading on March 31st, 2022 - Oral
6. The 2022 UKICRS Symposium, Barnes Wallis Building at the University of Manchester on 9th -10th June 2022 - Poster
7. PharmSci International Conference 2022, Belfast UK on 7th -9th September 2022 – Poster
8. MyCRS Young Scientist Symposium 2023, Kuala Lumpur, Malaysia on 17-18 August 2023 - Oral

List of publications from this thesis

1. Buang F, Chatzifragkou A, Amin MCIM, Khutoryanskiy VV. Synthesis of Methacryloylated Hydroxyethylcellulose and Development of Mucoadhesive Wafers for Buccal Drug Delivery. Polymers 2022;15:93. https://doi.org/10.3390/polym15010093. **Published (Chapter 3)**
2. Buang F, Fu M, Chatzifragkou A, Amin MCIM, Khutoryanskiy VV. Hydroxyethyl cellulose functionalised with maleimide groups as a new excipient with enhanced mucoadhesive properties. International Journal of Pharmaceutics 2023;642:123113. https://doi.org/10.1016/j.ijpharm.2023.123113. **Published (Chapter 4)**
3. Buang F, Cranwell P, Chatzifragkou A, Amin MCIM and Khutoryanskiy VV. Synthesis of acryloylated hydroxyethyl cellulose (HEC) as a new polymer with enhanced mucoadhesive properties. 2023. **In preparation for submission (Chapter 5)**
4. Buang F, Chatzifragkou A, Amin MCIM, Al- Obaidi H & Khutoryanskiy VV. Vinyl sulfone functionalised hydroxyethyl cellulose as a new excipient with enhanced mucoadhesive properties. 2023. **In preparation for submission (Chapter 6)**

List of publications not from this thesis

1. Shan X, Aspinall S, Kaldybekov DB, Buang F, Williams AC, Khutoryanskiy VV. Synthesis and Evaluation of Methacrylated Poly(2-ethyl-2-oxazoline) as a Mucoadhesive Polymer for Nasal Drug Delivery. ACS Applied Polymer Materials 2021;3:5882–92. https://doi.org/10.1021/acsapm.1c01097.

List of chapters

Chapter 1: Introduction

A concise introduction to the aims of the research, the main research questions and how these are addressed in the chapters of the thesis.

Chapter 2: Literature Review: Cellulose-based mucoadhesive polymers as multifunctional pharmaceutical excipients

A detailed review of the main background literature and how this led to the thesis project. In particular, the literature review chapters reviewed a large amount of literature on the topic and summarised the key findings that are important to the thesis.

Chapter 3: Methacryloylated HEC (HECGMA): Synthesis of methacryloylated hydroxyethyl cellulose and development of mucoadhesive wafers for buccal drug delivery.

The paper from this chapter was accepted and published in Polymers (MDPI) in 2023. In this chapter, glycidyl methacrylate was utilised as the source of the methacryloyl group, which is different from previous methacyloylation studies This chapter addresses the synthesis, characterisation, toxicity, development of a blank wafer model and investigation of the mucoadhesion of synthesised HECGMA.

Chapter 4: Maleimided-HEC (HECMAL): Hydroxyethyl cellulose (HEC) functionalised with maleimide groups as a new excipient with improved mucoadhesion properties.

The paper from this chapter was published in the International Journal of Pharmaceutics (Elsevier) in 2023. The modification work used N-(4-bromophenyl) maleimide, which is the first time this has been reported for chemically modified cellulose. This chapter covers the synthesis, characterisation, toxicity, development of a model for spray-coated tablets and study of mucoadhesion of synthesised HECMAL.

Chapter 5: Acryloylated HEC (HECAC): Synthesis of acryloylated hydroxyethyl cellulose (HEC) as a new polymer with improved mucoadhesive properties.

Acryloyl chloride was the reagent used in this modification work, and the synthesis was performed with non-aqueous trifluoroacetic acid as a solvent This chapter covers the synthesis, characterisation, toxicity, development of a blank film model and study of mucoadhesion of synthesised HECAC.

Chapter 6: Vinyl sulfoned HEC (HECVS): Vinyl sulfone functionalised hydroxyethyl cellulose as a new excipient with enhanced mucoadhesive properties

This chapter reports the successful modification of HEC with divinyl sulfone without cross-linking by changing the molar ratio of HEC to DVS. This chapter covers the synthesis, characterisation, toxicity, the development of blank microparticles model and mucoadhesion study of the synthesised HECVS.

Chapter 7: Concluding remarks and future work

Here the main findings of the research are summarised and critically discussed and suggestions for future work are set out.

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List of Abbreviations

^{1}H NMR	proton nuclear magnetic resonance
3-MPA	3-Mercaptopropionic acid
4-N-BPM	4-(N-Bromophenyl) maleimide
AC	acryloyl chloride
AFM	atomic force microscopy
AGU	anhydroglucopyranose
AHU	anhydroglucose unit
API	active pharmaceutical ingredient
APW	artificial pond water
BAC	benzalkonium chloride
$CaCl_2$	calcium chloride
Caco-2	cancer coli-2 “colon cancer”
CHI	chitosan
CHI High	chitosan high molecular weight
CHI Low	chitosan low molecular weight
CHI Medium	chitosan medium molecular weight
CMC	carboxymethylcellulose
CNC	cellulose nanocrystal
CNF	cellulose nanofibrils
COVID-19	coronavirus disease of 2019
CPP	cell-penetrating peptides
D_2O	deuterium oxide
DAD	Diode array detector
DCC	dicyclohexylcarbodiimide
Dex	dextran
DIW	deionized water
DLS	dynamic light scattering
DMAP	4-(dimethylamino)pyridine
DMF	N, N-dimethylformamide
DMSO-d6	deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPA	dopamine

DP	degree of polymerisation
DPTS	4-(Dimethylamino) pyridinium 4-toluenesulfonate
DS	degree of substitution
DSC	differential scanning calorimetry
EE	encapsulation efficiency
EMEA	European Medicines Agency
EPTMAC	2,3-epoxypropyltrimethylammonium chloride
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FTIR	fourier-transform infrared
GAG	Glycosaminoglycan
GMA	glycidyl methacrylate
GPC	gel permeation chromatography
GTAC	glycidyl trimethyl ammonium chloride
H_2SO_4	sulfuric acid
HA	hyaluronic acid
HCl	hydrochloric acid
HEC	hydroxylethyl cellulose
HECAC	modified acryloylated HEC
HECGMA	modified methacrylolated HEC
HECMAL	modified melamided HEC
HECVS	modified sulfoned HEC
HECGAC high	modified acryloylated HEC at molar ratio [HEC]:[AC] of [1]:[3]
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HECGMA medium	modified methacryloylated HEC at molar ratio [HEC]:[GMA] of [1]:[2]
HECGMAL high	modified melamided HEC at molar ratio [HEC]:[4-N-BPM] of [1]:[3]
HECGMAL low	modified melamided HEC at molar ratio [HEC]:[4-N-BPM] of [1]:[1]
HECGMAL medium	modified melamided HEC at molar ratio [HEC]:[4-N-BPM] of [1]:[2]
HECVS high	modified vinyl sulfoned HEC at molar ratio [HEC]:[DVS] of [1]:[0.9]
HECVS low	modified vinyl sulfoned HEC at molar ratio [HEC]:[DVS] of [1]:[0.1]

HECVS medium	modified vinyl sulfoned HEC at molar ratio [HEC]:[DVS] of [1]:[0.3]
HEK 293	human embryonic kidney
HGF	human gingival fibroblast cells
HPC	hydroxypropylcellulose
HPLC	High Performance Liquid Chromatography
HPMC	hydroxypropyl methylcellulose
IR	infra-red
IPEC	International Pharmaceutical Excipients Council
KCl	potassium chloride
kDa	kilo Dalton
LCST	lower critical solution temperature
LiCl	lithium chloride
LiOH	lithium hydroxide
MC	methylcellulose
Me_2SO	dimethyl sulfoxide
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MN	molar mass of nitrogen
MPF	maximum peak force
MS	moles of substituent
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2-5-Diphenyltetrazolium Bromide
NaCl	sodium chloride
NaOH	sodium hydroxide
NHDF	normal human dermal fibroblast
NP	nanoparticles
PAA	poly(acrylic acid)
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PEI	poly(2-ethyl-2-oxazoline)
PEMP	pentaerythritol tetrakis(3-mercaptopropionate)
PEO	polyethylene oxide
PETA	pentaerythritol tetraacrylate
PLGA	poly(lactide-co-glycolide)

PTSA	p-toluenesulfonic acid
PVP	poly(vinylpyrrolidone)
QUAD	Quantitative Uniform Authorship Declaration
RNA	ribonucleic acid
SANS	small angle neutron scattering
SAXS	small angle X-ray scattering
SEM	scanning electron microscopy
SH	Sulfhydryl
TA	tannic acid
TAB	tributyl ammonium bromide
TEA	Triethylamine
TEM	transmission electron microscopy
TFA	trifluoracetic acid
TGA	thermogravimetric analysis
TWA	total work of adhesion
UMU C3	epithelial like cell-isolated form urinary bladder
UPW	ultra-pure water
USP	U.S Pharmacopeial Convention
VSC	vinyl sulfone cysteamine
vWF	vonWillebrand factor
WHO	World Health Organization
XRD	x-ray diffraction
µcg	Microgram

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Chapter 1.

Introduction

Chapter 1.
Introduction

1.1 Aims and objectives

Polymers are large molecules composed of many repeating subunits. We encountered polymers almost every day in our life. They are found in both natural and synthetic forms and have a range of desirable properties that make them useful for many applications. Commercially available polymers have been prepared by modifying natural polymers to create new biomaterials with improved properties. For instance, modifications can be made to alter the polymer's solubility, biocompatibility, and mechanical properties, among others.

Hydroxyethyl cellulose (HEC) is a water-soluble and non-ionic cellulose derivative. In pharmaceutical formulations, HEC is widely used as an excellent thickening, stabilizing, and film-forming excipient. HEC dissolves easily in water, making it a versatile excipient. Despite its versatility, HEC has poor mucoadhesive strength for transmucosal delivery compared to charged polymers. This is because non-ionic polymer binds on weaker hydrogen bonds and therefore adheres to mucus more poorly than ionic and covalent bonds.

To overcome this limitation, there is a growing interest in modifying HEC to improve its mucoadhesive properties for transmucosal delivery. HEC has numerous hydroxyl (-OH) groups in its molecular structure. These hydroxyl groups make HEC highly reactive and suitable for chemical modification. One of the strategies is to modify HEC by introducing an unsaturated group such as methacryloyl, maleimide, acryloyl, and vinyl sulfone-containing molecules to the backbone structure of HEC. The molecules with unsaturated end groups will covalently attach to cysteine glycoprotein or thiol (a molecule containing a sulfhydryl group, -SH), in a reaction termed Michael addition reaction. This new multifunctional excipient may also promote great compatibility with charged drugs as the non-ionic part of HEC remains despite being mucoadhesive.

Therefore, the objective of this work is to synthesis and characterise modified HEC with methacryloyl, maleimide, acryloyl and divinyl sulfone and investigate their potential as a mucoadhesive polymer. Specifically, the work will focus on the modification of HEC with these unsaturated groups to improve the mucoadhesive properties of unmodified HEC. The specific objectives are outlined below, and Figure 1.1 summarizes the content of this thesis.

1. Modify HEC with methacryloyl, maleimide, acryloyl and divinyl sulfone-containing molecules.
2. Characterise the chemical properties of the modified HEC
3. Prepare a model blank dosage carrier of the modified HEC
4. Determine the mucoadhesive properties of modified HEC
5. Evaluate the toxicity of the modified HEC

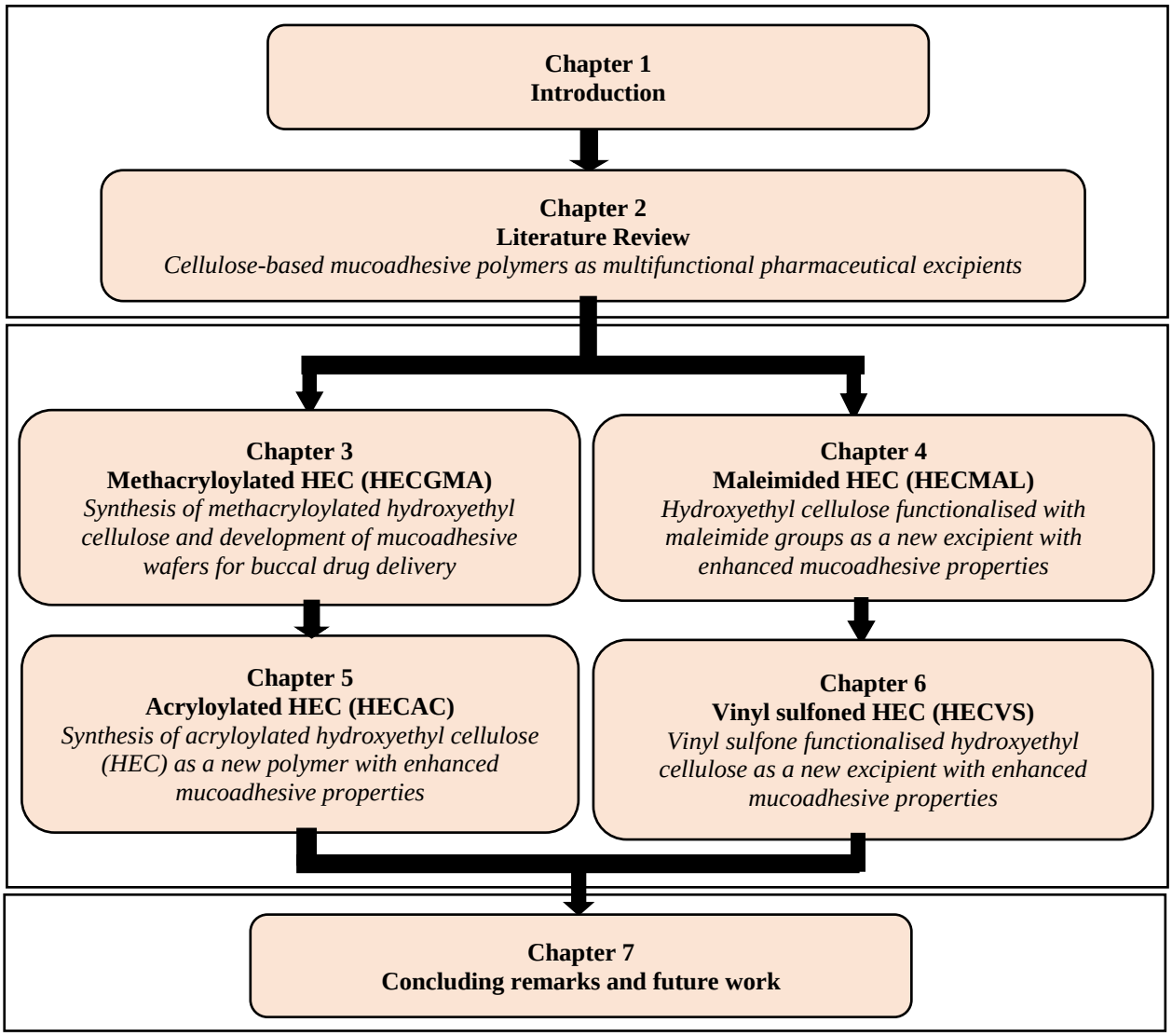

Figure 1.1. Summary of chapters in this thesis

1.2 New multifunctional excipients

The importance of excipients in pharmaceutical manufacturing has been highlighted in recent years. By 2025, the global market for pharmaceutical excipients is estimated to exceed $9.5 billion [1]. According to the European Medicines Agency website, excipients in medicine are substances other than the active pharmaceutical ingredient (API) that are added to a medicine to aid in its formulation, stability, and delivery [2]. Excipients are important to achieve formulation stability, lowering costs, increasing production efficiency, and producing a stable dosage form that is unaffected by changes in process settings or other constituents [3].

The International Pharmaceutical Excipients Council (IPEC), an international industry group established in 1991 has listed excipients by function, but not limited to binders, disintegrants, fillers, thickeners, lubricants, glidants, compression aids, colourants, sweeteners, preservatives, suspending /dispersing agents, film formers/coatings, flavours etc [4].

Excipients can generally be categorised by (i) modified excipients (physical or chemical modifications); (ii) co-processed excipients and (iii) novel excipients (new chemical entities) [5–7]. Modified and co-processed excipients are also classified by IPEC as 'new chemical excipients' based on safety data [8]. Excipients become multifunctional when they acquired additional functions besides their basic properties. Pharmaceutical manufacturers are interested in multifunctional excipients because they can solve multiple technical problems while improving formulation [1].

Recently, there is an increase in the development of new drug delivery methods which require new excipients that are compatible with the process [9]. New excipients offer opportunities and can have a significant impact on reducing the cost of manufacturing the drug or increasing its quality and safety [10]. In a survey conducted by the USP, 84% of respondents indicated that excipients currently in use hinder drug development, because (i) FDA-approved drugs in the chosen dosage form do not contain the excipient listed in current used or (ii) formulators have been unable to overcome challenges related to stability, bioavailability or solubility/permeability [11]. While for transmucosal delivery, the excipients lack adherence to the mucosal surface or site of release for prolonged drug release [12].

To overcome this, there is a need for the development of novel excipients. However, the demanding regulatory processes, long development times, expensive R&D investments, and the possibility of failure have discouraged manufacturers from producing novel excipients, thereby impeding market growth [9,13]. According to FDA guidelines, it takes almost two years to complete the toxicological data for novel excipients [14].

Therefore, developing a new modified excipient is preferred as it has the advantage of low R&D investment and low risk compared to co-processed (2-5 years) and novel excipients (6-7 years) [10]. This is because developing a newly modified excipient is less risky as it has a pre-approved functional purpose in a medicinal product and pharmacopoeia monograph [10]

1.3 Thiol-ene chemistry

The basis of my research is based on a 2010 study by professor Bianco-Peled's group, who presented a novel mucoadhesion system of sulfhydryl- acrylate interaction which is based on the ability of molecules that have an electronegative acrylate end group to form covalent bond with electronegative neighbouring groups, in a process referred as Michael addition [15]. This reaction can occur in a physiological environment without an initiator. Thus, the sulfhydryl or thiol (-SH) functional group from cysteine mucin and acrylate groups from a polymer can react via Michael addition to achieve strong mucoadhesion. There are several papers further discussing this theory to develop a mucoadhesive system between cysteine and substrate such as maleimide end group of EMC-5-FU prodrug [16], acrylated chitosan [17], 6-maleimidohexanoic acid-grafted chitosan [18], methacrylated poly (2-ethyl-2-oxazoline) [19] and acrylated poly (ethylene glycol)–alginate [20].

This reaction is categorised as a click chemistry reaction, introduced by Sharpless et al. in 2001. It describes the principle of efficient, simple organic synthesis that is rapid, has no or safe by-products, uses readily available reagents, insensitive to water and oxygen, and exhibits high efficiency under a variety of mild conditions [21,22]. The thiol-ene is one of the reactions of click chemistry. In general, it is a chemical reaction between a thiol (a compound containing a sulphur-hydrogen bond) and an unsaturated compound (a compound containing a carbon-carbon double bond/alkene) .

Two potential mechanism for thiol-ene click reaction is the thiol-ene radical and thiol Michael addition. In thiol-ene radical reaction, the alkene is usually activated by an initiator, e.g. a radical initiator or a photodynamic initiator, which triggers the formation of a radical on the alkene [23]. While the thiol Michael addition can be catalysed by a base or a nucleophile if the alkene is part of a Michael acceptor system [24]. Figure 1.2 shows the general mechanism of the thiol-ene click reaction.

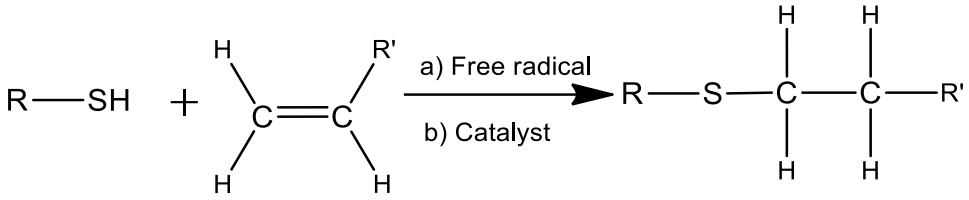

Figure 1.2. General mechanism of a thiol-ene click reaction.
(a) free radical (b) base- or nucleophile-catalysed [23]

Thiol-Michael addition reaction can be catalysed either by base or nucleophile with slightly different mechanisms. Generally, thiols and any terminal ene (a type of alkene) which are electron-poor unsaturated groups such as methacryloyls, maleimides, acryloyls and vinyl sulfones are able to participate in a chemical reaction of both base and nucleophile-mediated Michael addition [25]. In the base-catalysed reaction, the base abstracts a proton from the thiol to generate a thiolate anion, which subsequently undergoes thiol-Michael addition [26]. The reactivity rate and yield of base catalysed thiol-Michael addition reaction depends on the strength and concentration of amine catalysts, pKa of thiol, steric accessibility of the thiol, electron-poor unsaturated groups coupled to the C=C bond, solvent polarity and pH [26,27]. This was shown a work by Hubbel et al, where the thiol-Michael addition reaction of cysteine to various electron-poor enes is typically achieved by working with pH of the buffered aqueous systems to slightly above neutral pH (~ 8) [28,29].

While in the nucleophile-mediated pathway, the initial product of the nucleophilic addition towards an electron-deficient unsaturated group is a carbon-centred anion that functions as a strong base and deprotonates a thiol to produce a thiolate anion [26]. The exact type of bond depends on the specific nucleophile used in the reaction. The bond formed could be a carbon-sulphur bond (C-S) if a thiolate nucleophile is used, or a sulphur-hydrogen bond (S-H) if a hydride nucleophile is used.

1.4 Oromucosal delivery

To demonstrate the use of the newly synthesised HEC, I have chosen oral administration as a route for delivery. This route was chosen because it is the most common and promising route for drug delivery, with an annual product growth rate of 9.2–9.8% and a market value of $5.9–6.06 billion by 2028 [30]. Oromucosal drug delivery is defined as the administration of drugs through the oral mucosa to achieve a local or systemic pharmacological effect. The benefits of administering drugs via this route include avoiding first-pass hepatic metabolism and

gastrointestinal drug degradation, being easily accessible for patient administration, and being suited for dosage form administration and removal [31–33]. Therefore, this route is suitable for elderly, paediatric and palliative patients [34].

An important factor in oromucosal delivery systems is that they are often developed to achieve a faster onset of drug action. However, drugs delivered via the oromucosal route such as melatonin tablet for sleeping disorder and Ondansetron for treating nausea and vomiting requires a slow release of active ingredients. This is to provide sustained and prolonged relief of the symptoms.

1.4.1 Anatomy and physiology of oral mucosa

Knowledge of the anatomy and physiology of the oral mucosa is fundamental to understanding how medications and excipients are administered through the oral mucosa. The oral cavity is part of the digestive system and consists of two separate areas, the vestibule (the space between the lips, cheeks or teeth) and the oral cavity proper as shown in Figure 1.3 [35,36]. The oral cavity proper is the medial region of the teeth. The roof of the cavity is formed by the hard palate in the anterior region and the soft palate in the posterior region. The floor of the oral cavity is formed by the mylohyoid muscles. The inner lining of the oral cavity is a mucous membrane, the oral mucosa (consisting of stratified squamous epithelium). The oral mucosa performs an important function as a barrier in the human body. Figure 1.4 illustrates a cross-section of the components of the oral mucosa (epithelium, lamina propria, submucosa).

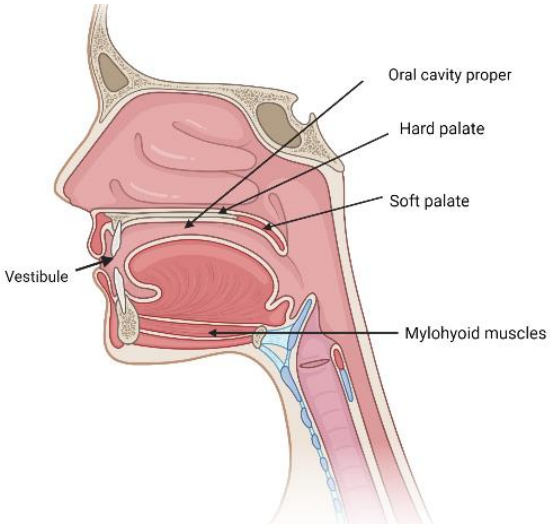

Figure 1.3. The oral cavity (sagittal section). Created with BioRender.com.

Based on Figure 1.4, the epithelium of the keratinised oral mucosa consists of four layers: (i) the stratum basale, (ii) the stratum spinosum, (iii) the stratum granulosum and (iv) the stratum corneum [31]. However, except for the dorsal surface of the tongue, the epithelium of the mucosa is covered by a non-keratinised epithelium. This epithelium varies in thickness depending on location, with the buccal mucosa area being the thickest at approximately 660 µm and the floor of the mouth at 100 µm [32]. It is estimated that the mucosa occupies 60% of the total surface area of the oral mucosa [33]. The remaining area is occupied 25% by the masticatory muscles and 15% by the specialised mucosa (dorsum of the tongue).

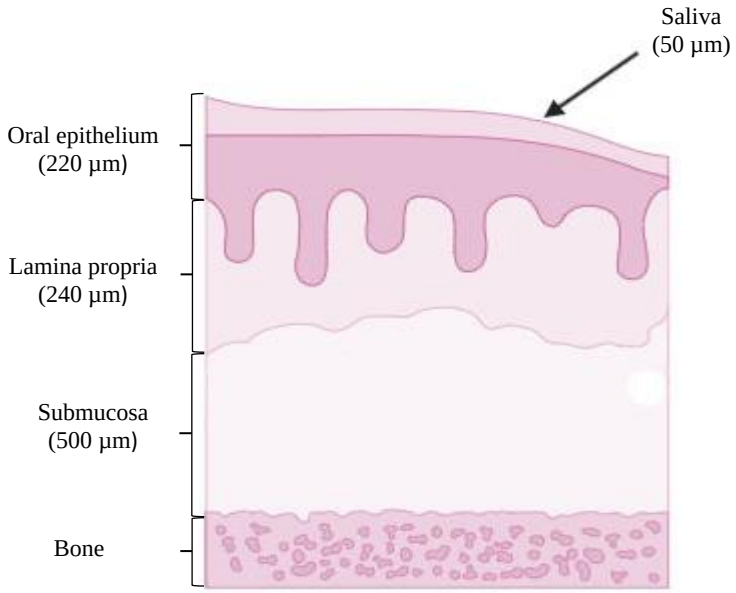

Figure 1.4. Cross section showing the components of oral buccal mucosa [37]. Created with BioRender.com.

The permeability of the oral mucosa is determined by its surface area and structure. When it comes to permeability, the epithelium acts as the primary barrier, and as the thickness of the tissue increases, so does the resistance of the barrier [34]. The gingival mucosa has the lowest permeability, followed by the buccal mucosa while the sublingual mucosa, often referred to as the floor of the mouth, is the region that is most easily penetrated [38].

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1.4.2 Mucin

The oral epithelium is protected by a layer of complex mucus, which not only provides lubrication but also promotes the flow of nutrients. Mucus is a highly hydrated gel composed of water (~95% w/w), mucins (~0.2 to 5.0% w/v), globular proteins (~0.5% w/v), salts (~0.5 to 1.0% w/w), lipids (1–2% w/w), DNA, cells and cell debris [39]. In the oral cavity, the mucus layer is not formed by mucus-secreting cells, but by mucins present in saliva [38]. It provides a protective layer by interacting with salivary proteins to alter their localisation and retention, which could enhance the protection of the oral cavity and reduce the pathogenicity of oral bacteria [40].

Nicolas Theodore de Saussure used the term "mucin" in 1835 to refer to substances extracted from mucus [41]. Mucins are glycoproteins and the most important structural components of mucus. They act as a barrier to protect underlying tissues from damage and infection, and they also help regulate fluid balance at mucosal surfaces [42]. Mucins can either be secreted or associated with the cell surface. Mucins that are secreted can be either water-soluble and of low molecular weight (200-300 kDa) or high molecular weight (over 1000 kDa) and gel-forming [43]. Gel-forming mucins are a major component of saliva (pH between 6.24 and 7.36). About 90% of all saliva is produced by the parotid, submandibular and sublingual glands, while the remaining 10% is produced by the minor salivary glands (labial, buccal and palatine) [44–46]. While saliva content is 99% water and 1% organic and inorganic compounds, the high molecular weight mucin has a carbohydrate content of 72%, a protein content of 18%, a sulphate content of 1.4% and a phosphate content of 1.45% [47].

There are several distinct regions in the structure of human salivary mucins consisting of a large central exon containing the entire tandem repeat domain, non-repetitive domains and cysteine-rich domains, as well as flanking 5′- and 3′-regions of vonWillebrand factor (vWF)-like domains (D-domains, the B-domain, the C-domain and the CK domain) [48].

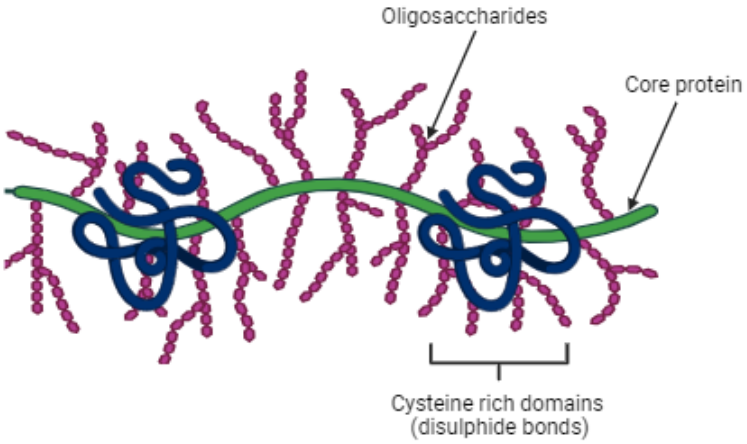

Figure 1.5. The structure of mucin glycoprotein [49]. Created with BioRender.com.

In general, the polypeptide backbone of mucin consists of serine, alanine, proline, glycine and threonine (75% of the total amino acid composition) [43]. The mucin protein backbone is also interspersed with cysteine domains stabilised by internal disulphide bonds, as shown in Figure 1.5 [39]. The C-knot or nested disulphide bond occurs when cysteine residues form covalent disulphide bonds with other cysteine residues, which contribute to the stability of the mucin structure and the formation of the mucus gel [50]. The knots are a structural motif consisting of three disulphides (6 cysteine residues in close proximity in a protein backbone) [50].

The two major mucins in human saliva are produced by the mucous cells of the submandibular glands, which produce the oligomeric mucin glycoprotein MUC5B (MG1), and by the serous cells, which produce the monomeric mucin glycoprotein MUC7 (MG2) [51]. In human saliva, the N-terminal domain of MUC5B (MG1) has about 450 amino acids rich in cysteine, while the N-terminal domain of MUC7 (MG2) contains two cysteine residues. Compared to MUC5B (MG1), the C-terminal domain of MG2 lacks a cysteine residue but is rich in proline [52].

In general, mucin is important for maintaining the health and integrity of mucosal surfaces in the body. Animal mucins, such as porcine gastric mucin and bovine submaxillary mucin, have no chemical differences from natural human mucin [38].

1.4.3. Oromucosal delivery systems

Oromucosal or oral mucosal administration is an effective method of systemic drug delivery that targets many mucosal regions of the oral cavity, including the buccal, sublingual, gingival,

palatal and labial mucosa (see Figure 1.6) [36], with the most common route of administration being the buccal and sublingual regions.

The therapeutic indication and mucosal region for administration usually dictate the dose form [30]. Before developing a dosage form, another factor to consider is the absorption of drugs on the site of application. In general, several factors influence the absorption of drugs via the buccal and sublingual areas.

1. Formulation residence time

   Neither route of administration is conducive to a prolonged residence time of a dosage form, as it is constantly cleansed by saliva and tongue movements. Depending on the formulation and the patient, the residence time varies and a longer time is needed to absorb the drug and achieve maximum systemic availability. One of the strategies is to incorporate mucoadhesive polymeric excipients into the formulation [53].

2. Drug absorption

   The membranes of oral epithelial cells are lipophilic, but the space between them is hydrophilic, resulting in hydrophilic and lipophilic zones [54]. To be completely absorbed via the sublingual route, a drug must have a balance between hydrophilic and lipophilic characteristics [12]. The drug must be soluble in aqueous buccal and sublingual fluids [12,55].

3. pH of the saliva

   Salivary pH can influence drug absorption by affecting the ionisation of the drug. Since the average pH of saliva is 6.0, this pH favours the absorption of drugs that are still ionised. In addition, drugs are absorbed through the oral mucosa when the pKa is greater than 2 for an acid and less than 10 for a base [12,55].

4. Flow of saliva

   The flow of saliva in the mouth increases when a substance is introduced into the oral cavity and depends on the taste, consistency and concentration of the substance. A swallowing reflex is triggered when the saliva volume is about 1.1 ml [44].

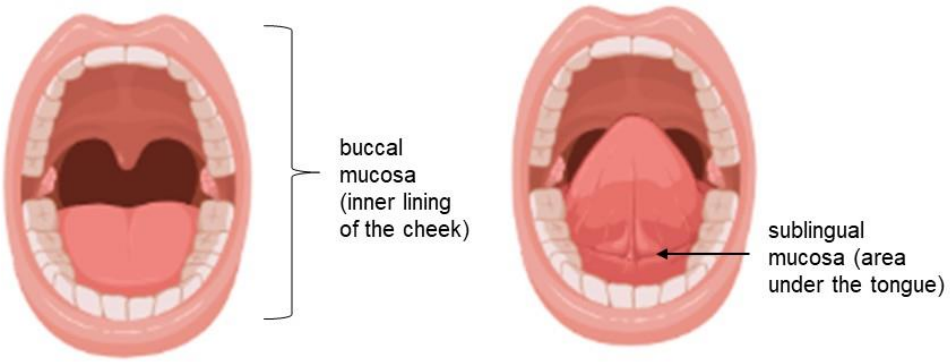

Figure 1.6. Schematic representation of oromucosal region. Created with BioRender.com.

1.4.3.1 Buccal delivery

Due to its large surface area, which accounts for approximately 23% of the total surface area of the oral mucosa (which includes the tongue), the buccal mucosa is suitable for the delivery of systemic drugs [38]. The approximate surface area covered by buccal membranes and other non-keratinized tissues is around 50 cm^2 of the 170 cm^2 total surface area of the oral cavity that are available for drug absorption [56].

Despite many advantages, buccal administration has several disadvantages, such as accidental swallowing of the dosage form, continuous dilution by saliva resulting in a low residence time of the formulation in the oral cavity, low bioavailability of the drug and low permeability of the buccal membrane compared to sublingual delivery [56–58].

Apart from that, developing dosage forms for buccal administration is challenging, as the maximum time for buccal administration of drugs is about 4-6 hours. Absorption of the drug may be affected as the epithelia of the oral mucosa change (3–8 days) more rapidly than that of the skin (30 days), altering the permeability properties over time [59]. Therefore, for buccal delivery, a 1-3 cm^2 device with a daily dose of 25 mg or less would typically be an appropriate delivery system.

1.4.3.2 Sublingual delivery

The sublingual mucosa is relatively permeable allowing for quick absorption and respectable bioavailability of the drugs. It is also a convenient and well accepted route by patients. This route has been the most researched due to its good permeability. The high permeability of the sublingual route is due to the thickness of the sublingual mucosa, which is between 100 and 200 µm, while the buccal mucosa is between 500 and 800 µm thick [60]. However, compared to buccal route (50.2 ± 2.9cm^2) the surface area of sublingual is small comprising only about 26.5 cm^2 ±4.2 cm^2 of the total oral mucosal surface area of 100-200 cm^2 [61].

However, this route of administration offers several advantages, including a faster onset of action, faster absorption of the drug and reduced side effects, as the medicine can be quickly discontinued by spitting out [12]. Most sublingually delivered drugs reach their peak blood levels within 10 to 15 minutes, which is typically a lot faster than when similar medications are taken orally [60].

Because the surface area of the sublingual region is much smaller, only small and rapidly dissolving dosage forms can be considered for this route [62]. Examples are drugs for the emergency treatment of angina pectoris, hypertension, treating cancer pain and migraine [62].

1.4.4. Dosage forms

Pharmaceutical dosage forms serve as a carrier for repeatable accurate dosing, quality, efficacy, safety, and stability, as well as to achieve high patient acceptance and compliance [8]. FDA-approved drugs for oral routes of administration are the most commonly available on the market with 62.02% from the total. This is followed by other routes of administration such as injection (22.43%), skin (8.70%), mucosal (5.22%), inhalation (1.21%) and other (0.42%).[65]. Most mucosal dosage forms currently on the market are solutions, sprays, tablets, ointments, creams and chewing gum, which account for 84% of the total [63]. There is indeed a growing need for dosage forms, especially for mucosal use, to provide patients with more options and improve treatment outcomes. A survey of 21 oromucosal products marketed in the USA and Europe found that 7 of these were tablets and 6 were lyophilisates/wafers. These are followed by films, sprays, powders and liquids [30].

For improved drug absorption and penetration, dosage forms for oromucosal delivery should ensure sufficient residence time of the formulation once it comes into contact with the oral mucosa under moist conditions [30]. For this purpose, excipients with mucoadhesive and permeability-enhancing properties are often used in the development of dosage forms.

1.4.4.1 Tablets

A mucoadhesive tablet is a type of oral drug delivery system that uses a mucoadhesive polymer to adhere to mucosal surfaces in the oral cavity. The physical characteristics of tablets are relatively small, flat and oval, with a diameter of approximately 5-8 mm [54,64]. Mucoadhesive tablets offer several advantages over conventional oral dosage forms. For example, they can be placed in different parts of the oral cavity and remain in the same place until dissolution [54].

Medications delivered via mucoadhesive tablets include anti-inflammatory agents, analgesics and antiviral agents. The availability of these products may vary by country and

region. Examples of commercial tablets for buccal and sublingual delivery include buprenorphine hydrochloride/naloxone hydrochloride dihydrate sublingual tablets (Zulsolv®) [65], fentanyl citrate buccal tablets (Fentora®) [66] and buprenorphine mucoadhesive sublingual tablets (Buprenex®) [67].

1.4.4.2.Films

An example of commercial films for buccal delivery is buprenorphine buccal film (Belbuca®). The BioErodible MucoAdhesive® technology is used to adhere the small, bilayered buprenorphine buccal film to the buccal mucosa. The buprenorphine buccal film is preferred over the transdermal drug delivery method for treating chronic pain because of its higher bioavailability (46–65%) and wider dosing range (75–900 g) [68].

In 2014, the buprenorphine/naloxone buccal film (Bunavail®) was introduced, which has a twofold higher bioavailability as it is absorbed in about half the buprenorphine dose, similar to (Suboxone®) (buprenorphine/naloxone) sublingual tablets. The dissolution time is 30 minutes, which is longer than buprenorphine/naloxone tablets (Zubsolv®) at 5 minutes [69]. The formulation uses a similar mucoadhesive system known as BioErodible MucoAdhesive (BEMA®) technology.

1.4.4.3 Microparticles/nanoparticles

Microspheres are defined as solid, approximately spherical particles with a diameter of 1 to 1000 μm [70]. The advantage of microcarriers over nanoparticles is that they act locally and do not migrate beyond the size of 100 nm through the interstitium carried by the lymph [71]. The physical properties of microparticles allow them to provide close contact with a larger mucosal surface better than other solid dosage forms [56]. Microspheres can be prepared by various methods, such as wax coating and hot melt, spray drying, coacervation, solvent evaporation, precipitation, freeze drying, single emulsion solvent evaporation, double emulsification and ionic gelation method [70].

The mucoadhesive polymer helps the microparticles to adhere to mucosal surfaces, such as those found in the oral cavity, respiratory tract, or female reproductive tract. By adhering to the mucosal surface, mucoadhesive microparticles can prolong the residence time of the drug at the site of action, resulting in better bioavailability and efficacy of the drug. Examples of drugs delivered via mucoadhesive microparticles include insulin (Afrezza®) [72] and budesonide (Pulmicort®) [73] etc.

1.4.4.4 Wafers

Oral wafers are solid dosage forms that dissolve quickly on the tongue without the need for water or chewing [74]. Thin oral wafers/oro-dispersible wafer strips are paper-thin with an area of 2-8 cm^2 and a thickness of 20-500 µm, typically containing less than 50 mg of active ingredients [75]. A thin oral wafer is usually designed for a fast-release system, which disintegrates in about 30 seconds [76].

The lyophilization method, in which polymer gels or suspensions are freeze-dried, can also be used to produce thick wafers with highly porous solid matrix up to 3 mm in thickness and 9 mm x 12 mm in size [76]. These wafers are suitable for extended release but must be designed to be mucoadhesive to allow a longer swelling time in the oral cavity.

Wafermine™, Wafesil™ and Xativa™ are examples of commercial wafers available on the market. All products are manufactured by iX Biopharma Pty Ltd, Australia, and are intended to be ingested by placing them under the tongue until they dissolve. The company uses WaferiX® technology, a patented sublingual wafer technology, to commercially manufacture wafers with proprietary formulations for both dietary supplements and pharmaceutical products. Wafesil, for example, is produced as an oval wafer available with 25 mg or 50 mg of sildenafil (as citrate) in the formulation.

1.4.4.5.Semisolid (ointments/gels)

Semi-solid, mucoadhesive dosage forms such as ointments and gels are formulated to adhere to the mucosal surface for a prolonged period. The mucoadhesive properties of these semisolid systems can be achieved by incorporating mucoadhesive polymers such as hydroxyethyl cellulose, carbomer or polycarbophil into the formulation. These polymers can interact with the mucous layer on the surface of the mucous membranes, thereby prolonging the residence time of the drug at the target site. Examples of prepared mucoadhesive semi-solid drug delivery systems include Adapalene, a topical retinoid gel for vaginal delivery [77] and Oral B pain relief gel [78] among others.

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Chapter 2. Literature Review

Cellulose-based mucoadhesive polymers as multifunctional pharmaceutical excipients

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Cellulose-based mucoadhesive polymers as multifunctional pharmaceutical excipients

Abstract

The global market for cellulose and its derivatives is expected to witness significant growth, directly attributed to the expansion of polymer applications in various fields. Cellulose is one of the naturally occurring polymers that is commercially used as a pharmaceutical grade polymer. Cellulose can be obtained from a variety of sources, including plants, animals, microorganisms and marine algae. As a versatile polymer, cellulose is of interest to many researchers and scientists to transform its original function into a multifunctional polymer. Over the years, the number of publications on mucoadhesion and cellulose as an excipient has increased. Cellulose has inherently poor mucoadhesive properties. Various strategies are used to improve the mucoadhesive properties of cellulose including (i) blending with other mucoadhesive polymers, (ii) modification into a charged polymer, (iii) modification with groups that specifically bind to mucosal tissue, etc. In this review, we discuss the necessary understanding of existing techniques and innovative systems for the use of cellulose and cellulose derivatives as excipients in mucosal drug delivery.

Keywords: mucoadhesion, cellulose, cellulose derivatives, excipient, modification

2.1. Introduction

Polymers are indispensable in our daily lives. They are large macromolecules formed from small monomer units by various reactions. Polymers that are of natural origin are called biopolymers. These polymers are naturally synthesised during the growth cycles of all organisms [1]. The most abundant biopolymer in our environment is cellulose. When Anselm Payen discovered cellulose in 1838, it was referred to as plant pulp or plant tissue for many years. [2]. Since then, cellulose has been further explored and well-studied by many researchers.

The industry uses plant-based cellulose extensively, mainly from wood pulp and cotton. According to FAO, nearly 186 million tonnes of cellulose will be produced globally in 2020, down 2% from 2019. Competition between material, energy and food for cellulose in nature is slowly depleting the plant-based cellulose source. However, cellulose can also be obtained from many other resources, such as plants (e.g. wood), animals (e.g., tunic of sea squirts-ascidians) microorganisms (e.g. bacterial cellulose) and marine algae (e.g. brown seaweed).

Cellulose and its derivatives are mainly used as excipients in the pharmaceutical industry. In pharmaceutical dosage form, it is used as a binder, lubricant, viscosity enhancer, gelling agent, etc. One of the additional properties that are of great interest in developing new cellulose-based excipients is mucoadhesion. Mucoadhesion is defined as a process in which macromolecules (synthetic or natural) adhere to the mucosal surface. These polymers are useful to prolong the drug residence time on the target site.

Currently, there are a considerable number of research and review papers on natural cellulose and its derivatives. The different types and categories of mucoadhesive polymers have been extensively reviewed previously [3-5]. However, to our knowledge, there is a limited comprehensive review on cellulose and mucoadhesion. In this review, we give a deeper insight into this topic as this would provide helpful information for future work on mucoadhesive polymers. This includes different strategies to improve the mucoadhesive properties of cellulose and cellulose derivatives as summarised in Figure 2.1.

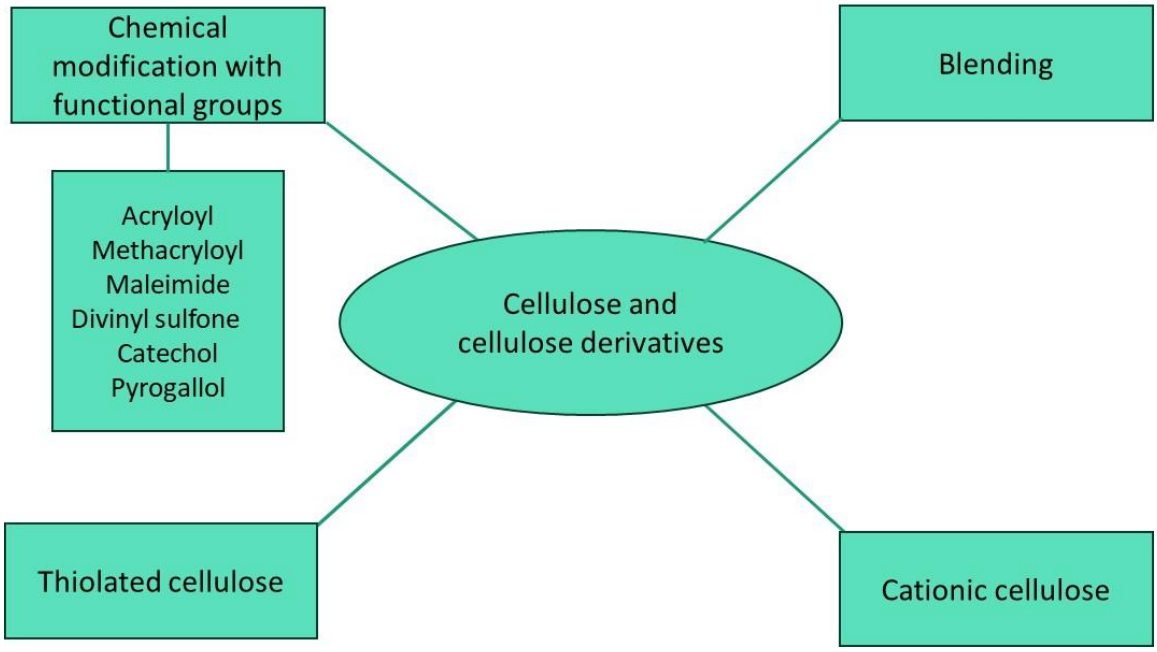

Figure 2.1: Strategies to improve the mucoadhesive properties of cellulose and cellulose derivatives.

2.1.1 The structure of cellulose

There are many books, reviews, and research papers describing the structure of cellulose in detail [6–11]. In general, cellulose is a homopolymeric polysaccharide with a linear long chain consisting of repeated D-anhydroglucopyranose (AGU) units. An AGU unit consists of one sugar structure. However, one repeating unit of cellulose consists of two sugars (disaccharides) called cellulobiose. It is connected by β -(1–4) linkages in opposite directions [12]. Thus, two alternately aligned AGUs of cellobiose are the basic monomer of cellulose. Since it is a long chain, the degree of polymerisation (DP) of cellulose is determined by the number of AGUs.

Each of these AGUs contains three hydroxyl (OH) groups located on the three carbon atoms of the cellulose backbone. The primary OH group is located at carbon 6, while the secondary OH groups are located at carbons 2 and 3. The reactivity of cellulose is determined by the availability of the three OH groups in the AGU [13].

Cellulosic materials offer a high specific surface area; therefore, surface modification helps to improve the performance and character of such materials. There are many possible approaches to modify cellulose-based materials.

The main characteristics of cellulose are that each of its repeating units carries three hydroxyl groups. Depending on the number of carbon atoms, the hydroxyl group at position 6 is the primary alcohol and at positions 2 and 3 are the secondary alcohol. This is due to steric

hindrance, with O-6 being more accessible and reported to react ten times faster than other OH groups [14]. However, in a homogeneous solution, the OH at position 2 is twice as reactive as at position 3 [15].

The OH group is polar and has an oxygen atom with high electronegativity. The extra electrons on the oxygen atom make the hydroxyl group nucleophilic. Therefore, reactions with electron-deficient molecules are easier. Thus, OH groups on cellulose can react with various molecules to facilitate the functionalisation of cellulose leading to various cellulose derivatives with multifunctional properties [16].

The macromolecules of cellulose are arranged in fibrils or bundles that form cellulose fibres. Cellulose fibres consist of two main components, which are amorphous and crystalline. These microfibrils are arranged as crystalline components in a high order of microfibrils. However, when the order of microfibrils is lower, it is called amorphous. Amorphous materials have no definite shape or formlessness [17].

2.1.2. The physicochemical properties of cellulose and cellulose derivatives

The macromolecular structure of cellulose influences the physicochemical properties of this polymer. Although it is the most abundant polymer, cellulose has a limitation. It is a polymer with high hydrophilicity. In its natural form, it is not water-soluble due to the strong intramolecular and intermolecular hydrogen bonds between the individual chains with a high degree of crystallinity (about 40-60 %) [18]. The hydrogen bonds can be broken by using suitable solvents such as solution of NaOH and LiOH [19,20], cadoxen [5], and dimethyl sulphoxide (Me_2SO) [6] or Me_2SO with LiCl [7]. There are also reports on the use of a combination of cellulose dissolving solvents at low temperatures, such as NaOH/urea, NaOH/thiourea and LiOH/urea aqueous solutions [21].

The reactions used for cellulose derivatisation are carried out under either heterogeneous or homogeneous conditions. A homogeneous modification process requires the complete dissolution of the cellulose in a suitable solvent. The dissolution of a polymer in a solvent involves two transport processes which are solvent diffusion and chain disentanglement [22]. When cellulose is dissolved in this solvent, the strong hydrogen bonding between the cellulose and the solvent molecules lowers the energy of the system; however, the exposure of the non-polar backbone of the cellulose in the polar solvent increases the energy of the system. This could be one of the most important reasons why NaOH is strong enough to break the hydrogen bonds of cellulose but has a limited ability to dissolve cellulose [19].

In the case of the water-soluble cellulose derivatives hydroxyethyl cellulose (HEC), the hydroxyethyl group probably contributes to the separation of the cellulose chains. It prevents the formation of hydrogen bonds between cellulose and enables dissolution in water [23]. Many etherification reactions of cellulose have been carried out in aqueous NaOH/urea solutions [21].

In pharmaceutical applications, cellulose is commercially used as an excipient in various drug formulations [24] Two of the most common modification process in cellulose is esterification and etherification. Water-soluble cellulose ethers are very versatile polymers, and their properties depend on several factors such as degree of substitution, molar substitution and others [25]. Examples of mostly used cellulose ethers are;

Methyl cellulose (MC) is a hydrophilic cellulose derivative used as a stabiliser [26], thickener [27], and emulsifier for food and cosmetics. In general, the manufacturing process of MC can be divided into two steps, alkalinisation and etherification. The hydroxyl groups (-OH) on the anhydroglucose monomers of the cellulose chain are partially replaced by methoxide groups (-OCH_3) after etherification.

The degree of substitution (DS) affects the solubility of MC in water or other solvents. DS of 1.6 -1.8 results in solubility in cold water but not in hot water [28]. MC has a thermal behaviour that acts as a lower critical solution temperature (LCST) polymer [29]. At a temperature higher than 29 ± 2 °C, it forms a thermoreversible gel [27,29]. Due to this unique property, MC has been widely used as a hydrogel for drug delivery, e.g., as a wound dressing by freeze-drying [30], as an *in situ* ophthalmic gel [31] or as a gel for enteral administration [32], etc.

Hydroxypropyl cellulose (HPC) is a product of the etherification of cellulose with propylene oxide [33]. It has thermoresponsive properties similar to MC [34]. It has LCST behaviour at a temperature of 41-45 ˚C [35]. Therefore, HPC has been used in drug delivery as a microsphere hydrogel with other polymers such as chitosan, acrylamine-grafted HPC and chitosan [36,37].

Carboxymethyl cellulose (CMC) is an anionic, water-soluble cellulose derivative with carboxymethyl groups (i.e., –CH2COOH) replacing the hydroxyl group in the cellulose backbone [38]. The carboxymethylation process involves the etherification of pure cellulose under heterogeneous conditions. The alkaline condition is important for determining the properties of CMC (yield, lightness, crystallinity, etc) [39].

Hydroxyethyl cellulose (HEC) is a non-ionic cellulose derivative that is easily soluble in cold or hot water but not in most organic solvents. HEC is widely used as a thickener, binder,

emulsifier, suspending agent, dispersant, and stabiliser, which are used in a variety of applications. HEC powder is stable, although it is a hygroscopic material. Aqueous solutions of HEC are relatively stable at a pH of 2–12, and the viscosity of the solutions is hardly affected [40]. To produce HEC, cellulose pulp is soaked with sodium hydroxide and then reacted with gaseous ethylene oxide (etherification process). In this process, the hydrogen atom in the hydroxyl group of cellulose is replaced by a hydroxyethyl group [41].

The reaction of ethylene oxide with cellulose under optimised conditions and with optimised number of moles of (MS) leads to complete solubility of HEC in water. HEC with complete solubility in water has MS of 2.5. Natrosol 250, a commercially available HEC, for example, has MS of 2.5 (5 ethylene oxide groups/2 AHU units) and a degree of substitution (DS) of 1.5 (3 hydroxyl groups/2 AHU units). An anhydroglucose unit (AHU) has three reactive hydroxyl groups. A complete substitution would therefore lead to a DS of 3 [42]. In drug administration, HEC has the advantage of higher water binding, twice that of methylcellulose and hydroxypropyl cellulose [43].

2.2. Mucoadhesion and transmucosal delivery

The term bioadhesion in drug delivery refers to the adhesion of materials to a biological surface and mucoadhesion is adhesion to mucosal surfaces [44]. The mucous membrane is a layer lining the wet surfaces in the human body, including the digestive, respiratory, and reproductive organs, and serves primarily as a protective barrier.

Mucus is known for its stickiness and adheres to the epithelial surface of the mucosa. In humans, the goblet cells or exocrine glands with mucous cells acini secrete mucus with an average thickness of about 50 -450 μm. The general composition of the mucus layer is 95% water, 0.5-5.0% glycoproteins and lipids, 0.5-1.0% mineral salts, and 0.5-1.0% free proteins [45]. The mucin glycoprotein, which contributes to the gelation of mucus, contains sialic acid and sulphate groups. The sialic acid and sulfate groups ensure that mucin is negatively charged (-10mV) at neutral pH. The molecular weight of mucins varies from 500 kDa to 20 MDa. Mucin also provides several H-bond donors and acceptors as well as ionic bonds with mucoadhesive materials [46]. According to the results of one study, porcine gastric mucus contains 2.62 ± 0.429 nmol thiol groups per mg [47].

Therefore, in the pharmaceutical field, mucosal surfaces are the potential route for drug delivery for systemic therapeutic effects, including oral (buccal and sublingual), vaginal, nasal, ocular, and rectal. Table 2.1 showed a list of some commercial mucoadhesive products available in the market while Table 2.2 showed a few of the many patents registered for

oromucosal administration. The advantages of mucosal delivery systems include an effective local site of action, rapid absorption of the drug and an increase in drug concentration when adhered to the mucosa [45]. It was also observed when there is an increase in surface area and blood flow helps in increasing the bioavailability of the drug and this will improve patient adherence to treatment as it reduces the frequency of medication intake [44]. Lastly, this route is one of the best options for administering drugs to palliative patients, especially during the COVID-19 pandemic that is affecting worldwide [48].

Two important surfaces are involved in mucoadhesion, namely the polymer and the mucosal surface. Mucoadhesion mechanism occurs in two stages which is contact and consolidation phase as shown in Figure 2.2 (a). When the polymer comes into contact with the mucosal surfaces (wetting), there is generally interpenetration of the polymer followed by a consolidation phase that strengthens the bond [49,50]. During the wetting and swelling phase, the mucoadhesive polymer is dispersed to improve the contact stage and increase the surface area. The polymer then diffuses into the mucus layer (interpenetration phase), and in the consolidation stage, binding occurs through mechanical/chemical interaction for prolonged adhesion, depending on the type of polymer [49–51].

The study of the mechanism of mucoadhesion is challenging as it involves numerous fields of science such as physical surface chemistry, materials science, etc. In the consolidation phase, the adhesive bond can be formed by physical or mechanical bonds (interpenetration, entanglement, and mechanical interlocking of mucoadhesive polymer chains and mucin glycoprotein chains), secondary chemical bonds (van der Waals forces and hydrogen bonds) and primary chemical bonds (covalent and ionic bonds) [52–55]. These forces are the basic knowledge of surface chemistry that is essential for understanding the mechanism of mucoadhesion. Several articles have extensively reviewed the different theories of mucoadhesion (electronic, wetting, adsorption, diffusion, and fracture theories) to fully understand the mechanism of mucoadhesion

Figure 2.2.(b) illustrated the different theories of mucoadhesion. The first theory electronic describes the charged double electrical layer forms when these two surfaces (bioadhesive polymer and biological surface) come into contact with one another due to electron transfer.

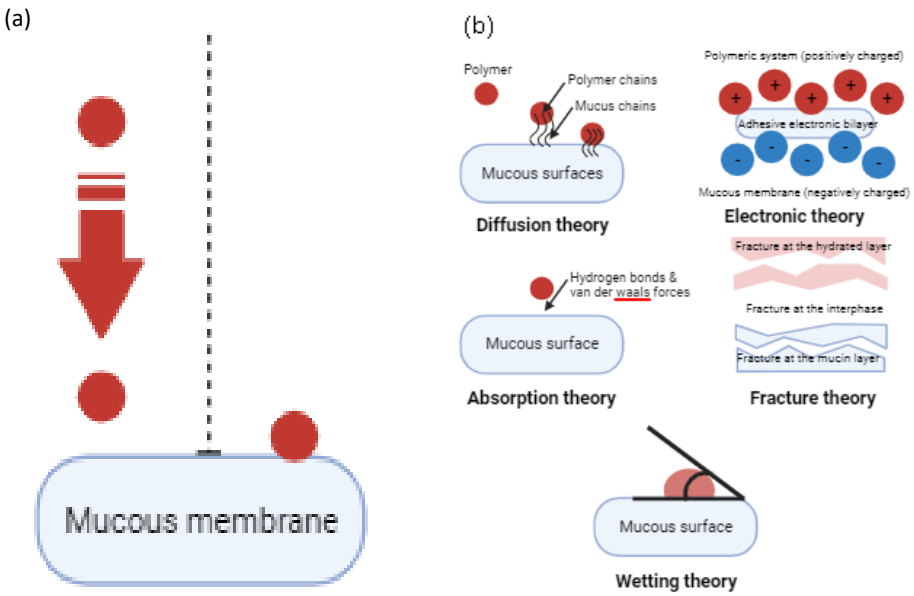

Figure 2.2: Mucoadhesive mechanism and theories. (a) two stages, contact and consolidation (b) mucoadhesive theories that explain the relationship between mucoadhesive surfaces and delivery systems [56]. Created with BioRender.com.

While wetting theory describes the interaction between a liquid and a mucoadhesive surface, which is mostly determined by the substance's contact angle with the surface. The adsorption describes the forces at the surfaces of atoms following initial contact. According to this theory, there are two different kinds of chemical bonds that might form: (a) a primary unfavorable covalent bond that may result in a permanent bond and (b) a secondary bond that is attracted by van der Waals, hydrogen bonds, electrostatic forces, and hydrophobic interactions. The main concept behind the diffusion theory is that mucus glycoproteins and mucoadhesive polymer chains entangle to form a semipermanent adhesive bond. The process can be seen starting at the first point of contact. Lastly the fracture theory describes the force required to break the bond between the two materials following mucoadhesion. [49,53,57].

When adhesion occurs, a single theory for mucoadhesion may be insufficient to explain the process. The mechanism by which mucoadhesive bonding occurs is determined by several factors, including the nature of the mucosa and mucoadhesive material, the type of formulation, the adhesion process and the subsequent environment of bonding [51].

Table 2.1. Examples of commercial transmucosal dosage forms licensed and used in the UK (https://www.medicines.org.uk/emc/)

Active ingredients	Brand	Company	Dosage strength	Pharmaceutical form
Fentanyl	Abstral®	Kyowa Kirin Ltd, Japan	100µg, 200µg, 300µg, 400µg, 600µg, 800µg	Sublingual tablet
	ACTIQ®	Teva Pharmaceutical Industries Ltd, Israel	200µg, 400µg, 600µg, 800µg,1200µg, 1600µg	Compressed lozenges with integral oromucosal applicator
	Effentora®	Teva Pharmaceutical Industries Ltd, Israel	100µg, 200µg, 400µg, 600µg, 800µg	Buccal tablet
	Instanyl®	Takeda Pharmaceutical Company Ltd, Japan	50µg, 100µg, 200µg	Nasal spray
	PecFent®	Kyowa Kirin Ltd, Japan	100µg, 400µg	Nasal spray
	Cynril®	Fontus Health Ltd, UK	200µg, 400µg, 600µg, 800µg,1200µg, 1600µg	Compressed lozenges with integral romucosal applicator
Extract from the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*	ACARIZAX	ALK-Abelló A/S, Denmark	12 SQ-HDM	Oral lyophilisate

Lidocaine Hydrochloride	Anbesol Adult strength	Alliance Pharmaceuticals Limited, UK	2% Lidocaine hydrochloride	Oromucosal gel
	Anbesol Teething gel		1% Lidocaine hydrochloride	Oromucosal gel
	Anbesol liquid		0.9% Lidocaine hydrochloride	Oromucosal liquid
Budesonide	Benacort®, Benacort® hayfever	McNeil Products Limited, USA	64 µg	Nasal spray
	Budelin® Novolizer®	Mylan Products Ltd, USA	200µg	Inhalation powder
Diclofenac sodium	Econac	Mercury Pharma Group Ltd, UK	100µg	suppositories
Extract of grass pollen from Timothy (*Phleum pratense*)	Grazax	ALK-Abelló A/S, Denmark	75,000 SQ-T	Oral lyophilisate
Meloxicam	Meloxicam	Alpex Pharma (UK) Limited, Switzerland	7.5mg, 15mg	Orodispersible tablet
Nicotine	Nicorette Cools	McNeil Products Limited, USA	2 mg, 4mg	Lozenges
	Nicorette QuickMist	McNeil Products Limited, USA	1 mg	Oromucosal spray
Mesalazine	Salofalk	Dr. Falk Pharma GmbH	1.0 g	Rectal foam

Suboxone	Suboxone	Indivior, USA	2 mg/0.5 mg film, 4 mg/1 mg film, 8 mg/2 mg film, 12 mg/3 mg	Sublingual film
Xylometazoline Hydrochloride	Sudafed	McNeil Products Limited, USA	1 mg /1ml of solution	Nasal spray

Table 2.2. Examples of patents granted on mucoadhesive pharmaceutical dosage forms for transmucosal delivery (https://www.wipo.int/patentscope/en/)

Patent no	Date of publication and grant	Title of invention	Inventor	Office	Field of invention/description
EP2646006	09.10.2013	A Pharmaceutical Dosage Form	Shaikh Rubina Perveen Pillay Viness Choonara Yahya Essop Du Toit Lisa Claire, *Univ Witwatersrand JHB*	European Patent Office	A pharmaceutical dosage form for releasing a pharmaceutically active ingredient includes a mucoadhesive layer that can adhere to the gastrointestinal tract or oral mucosa of humans or animals, a water-insoluble outer layer, and an intermediate layer that contains the active ingredient. The intermediate layer is disposed between the mucoadhesive and water-insoluble outer layers. The mucoadhesive layer may be made of polyacrylic acid, chitosan, pectin, HPC, HPMC, HEC, or other polymers.

WO2017024237	05.08.2016	Composition and method for treatment of metabolic disorders	Jozefiak, Thomas Parlato, Michael Patel, Pratik Colbert, Kevin Nimgaonkar, Ashish Pasricha, Pankaj, *The Johns Hopkins University [Us]/[Us]*	USA	The invention features mucin-interacting components and viscosity-modifying techniques. In one embodiment, CHI is formulated together, HEC, and Dex. In embodiments, CHI is used in the range of 0.1-10.0% (w/w), HEC is used in the range of 0.1-2.0%) (w/w) and Dex is used in the range of 0.05-1.0%) (w/w). In one embodiment, the CHI/HEC/Dex ratio is 3/0.5/0.3.
17.11.2022	US20220362195	Dimethyl fumarate particles and pharmaceutical compositions thereof	Pierre Boulas Erwin Irdam Shyam B. Karki William F. Kiesman Cheuk-Yui Leung Yiqing Lin Andrea Trementozzi Peter Zawaneh, *Biogen Ma Inc.*	USA	The present invention provides dimethyl fumarate (DMF) particles and methods for making them, as well as DMF-coated particles with an enteric coating. Mucoadhesive dosage forms may contain an active agent and one or more polymers. In some embodiments, the active pharmaceutical ingredient is sealed, enteric coated, or sealed and enteric coated.

US20070281007	06.12.2007	Mucoadhesive oral formulations of high permeability, high solubility drugs	Jacob Jules S. Moslemy Peyman Nangia Avinash Shaked Ze'ev Kreitz Mark	USA	The present invention provides a mucoadhesive solid oral dosage form closely attach to the target epithelium and help the medicine diffuse into intestinal tissue. The mucoadhesive polymer can be distributed in the tablet matrix or applied as a direct compressed coating. Preferred mucoadhesive polymers include poly(adipic acid) anhydride “P(AA)” and poly(fumaric acid-co-sebacic acid) anhydride “P(FA: SA)”. Other preferred mucoadhesive polymers include non-erodible polymers such as DOPA -maleic anhydride copolymer, isophthalic anhydride polymer, DOPA - methacrylate polymers and DOPA - cellulose-based polymers
WO2021100063	23.11.2020	Oral film composition comprising Levothyroxine	Aurora, Jack Syed, Moinuddin Rashid	India	The invention relates to a pharmaceutical composition in the form of an oral film comprising

			Gawali, Rajendra, *Wockhardt Limited [IN]/[IN]*		levothyroxine and a film-forming polymer. The invention also relates to a method of making such oral film compositions. the film-forming polymer is selected from one or more of (a) the cellulose derivatives are selected from one or more of ethyl cellulose, methylcellulose, HPMC, Methocel E15, Methocel K15, HPC, HEC, hydroxymethyl cellulose, hydroxypropylethyl cellulose, carboxymethylethyl cellulose, carboxyethyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose, etc.
US20210353529	18.11.2021	Mucoadhesive patch and uses thereof	Solaleh Miar Gregory Robert Dion Joo Leng Ong Teja Guda,	USA	The present invention provides a mucoadhesive patch for attachment to a mucosal surface in a patient. The patch comprising a fibrous polymeric mat substrate and a plurality

			Board of Regents, The University of Texas System		of polymeric flock particles attached to the substrate.
IL183753	29.12.2016	Multiparticulate form of administration comprising nucleic acid-containing mucoadhesive active ingredients and method for producing said form of administration	Reinhold Cohen and his partners, *Evonik Rohm GmBH*	Israel	The invention involves a multi-particulate pharmaceutical form with mucoadhesively formed nucleic acid active components and its production. The patch has two sides: one with low water or body fluid permeability, such as ethyl cellulose, and one with the active ingredient, such as a protein, polysaccharide, or small molecule, which may be mixed with mucoadhesive polymers like chitosan, CMC, polyacrylic acid, or pectin.

2.3. Cellulose and cellulose derivatives as mucoadhesive polymers

Cellulose and cellulose derivatives belong to the first-generation mucoadhesive polymers. The first-generation polymers lack targeting and specificity in adhering to mucus. [58,59]. The first-generation polymers can be further divided into cationic, anionic, and non-ionic polymers [60].

A key factor in the mucoadhesive properties of a polymer is its ability to form strong intermolecular hydrogen bonds, e.g., carboxyl (COOH), hydroxyl (OH), amide (NH_2), and sulphate (SO_4H) groups with the mucosal layer. In addition, the polymer should penetrate the mucosal network or tissue, wet the mucosal layer, and have a high molecular weight [61].

Cellulose ethers are cellulose derivatives with a high molecular weight and alkyl or substituted alkyl groups, that have been reported to improve bioadhesive strength. Non-ionic, water-soluble cellulose ethers such as HEC, HPC, MC or HPMC and anionic derivatives such as NaCMC are among the commonly used polymers for the preparation of mucoadhesive dosage forms [62]. Cellulose ethers have advantages for transmucosal formulation due to their ability to increase the viscosity of the aqueous solution, their bioadhesiveness, non-toxic nature, their low bioavailability and their ability to form films [63]. In a study by Ivarsson and Wahlgren, they compared six polymers, including cellulose derivatives, sodium CMC, and HEC, in terms of their mucoadhesive properties [64]. Using the tensile strength method (texture analyser), they found that HEC had the highest adhesive strength compared to the others.

In another study, it was found that the mucoadhesive properties of HPMC were higher than those of HEC and HPC [65]. When comparing polymers with a similar average molecular weight (e.g., HEC and HPC), no correlation was found between the average molecular weight of the polymer and the mucoadhesive properties. Apparently, different polymers have different optimal molecular weights for maximum mucoadhesive strength [66]. In general, polymers with a molecular weight greater than 100,000 kDa have higher mucoadhesive strength [45]. This is in contradiction with a study on HPMC with different grades, which found that mucoadhesive strength increased with increasing viscosity of HPMC [67]. They suggested that the viscosity of the polymer significantly affects mucoadhesive performance.

Therefore, non-ionic cellulose derivatives especially HEC typically possess mucoadhesive properties but perform poorly compared to charged polymers.

2.4. Strategies to improve the mucoadhesive properties of cellulose and cellulose derivatives

2.4.1. Chemical modification with functional groups

In 1833, Braconnot made the first attempt to chemically modify cellulose from a variety of cellulose materials to form cellulose nitrate [68]. There are several techniques of modification and changes to the outer layer of material called "surface modifications" [69]. Surface modification can be achieved by several methods such as physical, mechanical, chemical methods etc [70]. Here we discuss the modification of polymers, especially cellulose and its derivatives with different functional groups as a strategy to improve the mucoadhesive properties.

2.4.1.1.Acryloyl

In 2010, Bianco-Peled's group presented a new mucoadhesive system based on the potential of modified polymers with acrylate end groups to bind covalently to cysteine residues in mucin glycoproteins via the mechanism of Michael addition reaction [71]. This idea was developed by Hubbel and colleagues who successfully conjugated proteins with sulfhydryl biomolecules to unsaturated groups.

Conjugation of polymers with acryloyl groups has been reported to enhance the mucoadhesive properties of polymers. These reactive moieties can create covalent connections with cysteine, an amino acid building block of mucins.

We have summarised recent work on modified polymers with acryloyl groups in Table 2.3. It should be noted that we have not found any work on acryloylated cellulose for mucoadhesion. This could be because cellulose has poor mucoadhesive properties as it is not charged. But here are several works reported on the acryloylated modified cellulose with CNC [72], MCC [73] and CMC [74] for other applications. These works reported on the physical and chemical characterisation of modified cellulose.

2.4.1.2.Methacryloyl

Methacryloyl group have the same ability as acryloyl and maleimide groups to form covalent bonds with thiols under physiological conditions. Recently, the Khutoryanskiy group reported that methacrylation of chitosan, gellan gum and HEC leads to polymers with improved mucoadhesive properties. They discovered that the methacryloylated polymers were more mucoadhesive than the unmodified polymers [75–77].

A similar result was obtained in a study on methacrylated hexanoyl glycol chitosan which is a soluble chitosan derivative. In this study, it was found that the presence of methacrylated chitosan leads to a strong interaction with mucin. It was hypothesised that this leads to the formation of covalent bonds between the thiol group of mucins and the methacrylate moiety of methacrylated hexanoyl glycol chitosan [78].

Chemicals used to conjugate methacryoyl groups include methacryloyl chloride [79], methacrylic anhydride [80], and glycidyl methacrylate (GMA) [81–83]. GMA has a very reactive epoxide group compared to the ester category. It is suggested that a higher degree of modification can be achieved by reacting GMA in protic solvents [84]. In an aprotic solvent, polysaccharides reacted with GMA mainly by transesterification, whereas in protic solvents the reaction occurs by opening the epoxide ring.

In a report by Reis et al, the transesterification mechanism is a fast and reversible reaction pathway, while the epoxide ring opening mechanism is a slow and irreversible one [83]. Therefore, GMA is considered to be the more efficient compound for the methacrylation of cellulose and cellulose derivatives. Table 2.4 summarises recent work on the methacryloylation of polymers to improve mucoadhesion properties.

Table 2.3. List of polymers modified with acryloyl groups to improve the mucoadhesive properties

Primary polymer	Acryloyl group containing substance	Characterisation method	Quantification	Mucoadhesion method	Dosage form	Biological tissue	Toxicity assay	Reference
Chitosan 207kDa (Low MW)	PE-diacrylate 0.7kDa & 10kDa	^{1}H NMR (Solvent: 1% v/v CD_3COOD)	Ninhydrin test	Rotating cylinder,	Tablet	Porcine intestine	Nil	[85]
Chitosan 207kDa (Low MW)	PEG-diacrylate 10kDa	^{1}H NMR (Solvent: 2% v/v CD_3COOD + 98% v/v D_2O) & DLS with Zetasizer	Fluorescamine test	Retention study, Nanoparticels (NP)-mucin aggregation	NP	Porcine intestine	Nil	[86]
Eudrgagit EPO 135kDa	Acryloyl chloride	^{1}H NMR (Solvent: deuterated chloroform), FTIR & TGA	Elemental analysis & Back permanganometric titration	Retention study	Nil	Sheep nasal tissue	mucosal irritation test	[87]

Alginate	PEG-diacrylate 10kDa	^{1}H NMR (Solvent: D_2O)	Ellman's assay	Adhesion assay using tensile machine	Nil	Porcine small intestine	Cell viability (Neonatal human foreskin fibroblast)	[88]
Pentaerythritol tetrakis(3-mercaptopropionate) (PEMP)	Pentaerythritol tetraacrylate (PETA)	^{1}H NMR (Solvent: DMSO-d6), DLS with Zetasizer, TEM & FT-Raman spectroscopy	Ellman's assay	Retention study	NP	Porcine urinary bladder	Nil	[89]

Table 2.4. List of polymer modified with methacryloyl groups to improve the mucoadhesive properties

Primary polymer	Methacryloyl group containing substance	Characterisation method	Quantification	Mucoadhesion method	Dosage form	Biological tissues	Toxicity assay	Reference
Chitosan 370 kDa	Methacrylic anhydride	^{1}H NMR (Solvent: D_2O acidified with 30 µL trifluoroacetic acid), FTIR, Zetasizer, XRD	Degree of methacrylation using ^{1}H NMR & Ninhydrin test	Retention study	Nil	Porcine urinary bladder tissue	Cell viability (UMU C3 cell line)	[90]
Gellan gum ~ 1000 kDa	Methacrylic anhydride	^{1}H NMR (Solvent: D_2O), FTIR and Zetasizer	Degree of methacrylation using ^{1}H NMR	Retention study	Nil	Bovine conjunctival tissue	Nil	[91]
Poly(2-ethyl-2-oxazoline)	Methacrylic anhydride	^{1}H NMR (Solvent: D_2O & DMSO-d6), FTIR and DSC	Degree of methacrylation using ^{1}H NMR	Retention study	Nil	Sheep nasal septum mucosal tissue	Slug mucosal irritation study & Cell viability (HEK 293 cell line)	[92]

HEC 720 KDa	Glycidyl methacrylate	^{1}H NMR (Solvent: D2O) and FTIR	Degree of methacrylation using ^{1}H NMR and HPLC	Tensile test	Wafer	Sheep buccal tissue	Cell viability (Caco-2) and planaria toxicity and fluorescence intensity test	[76]

2.4.1.3.Maleimide

Maleimide is a heterocycle normally used as a photosensitive material. Maleimide functional group may act as a great mucoadhesive moiety due to its high selectivity and reactivity towards cysteine molecules found in mucin [92,93]. Similar to acryloyl and methacryloyl groups, maleimides can also react with unsaturated groups via the Michael additions

In most works on maleimides as mucoadhesive components, the polymers used are chitosan, polyethylene glycol (PEG), poly(vinylpyrrolidone) (PVP) and poly(lactide-co-glycolide) (PLGA) [93–95]. Recently, a modification of carboxymethyl cellulose with maleimide groups was carried out by reacting the N-substituted imide of furan-protected maleic anhydride with N-Boc-ethylenediamine, which exhibits strong mucoadhesive properties [96]. There is also work on the modification of cellulose acetate by N-(phenyl amino) maleimides to improve the thermal and mechanical properties of the polymer [97]. The reaction between the acetyl group of the glucopyranose ring in cellulose acetate and the proton of the amino group in the N-(phenyl amino) maleimide molecule is the basis for the chemical modification.

Maleimides are known to hydrolyse rapidly in neutral to alkaline environments [98]. The disadvantage of maleimide is its instability. It is easily hydrolysed and shows undesirable side reactions with amines at alkaline pH. Table 2.5 lists the polymers that have been modified with maleimide molecules to improve mucoadhesive properties.

2.4.1.4.Vinyl sulfone

Vinyl sulfone can be used both as a Michael acceptor and in cycloaddition reactions. Due to the high reactivity of vinyl sulfone towards a number of nucleophiles, a variety of modified polysaccharides can be prepared. [99].

The vinyl sulfone group is very stable in a wide pH range (from 4 to 10) [34] and can react with primary and secondary amine, hydroxyl, phenyl, thiol or imidazole groups under different pH conditions [100]. Divinyl sulfone (DVS) is a molecule reactive to nucleophiles with two active sites corresponding to the outer carbon of the double bond. The advantages of DVS are its high reactivity, its ability to work in an aqueous environment, and its low boiling point, which facilitates the removal of excess [101]. Divinyl sulfone has also been used to immobilise some enzymes [100], post-translational modification of proteins [102], and formation of hydrogels [103].

Table 2.5. List of polymers modified with maleimide groups to improve the mucoadhesive properties

Primary polymer	Maleimide group containing substance	Characterisation method	Quantification	Mucoadhesion method	Dosage form	Biological tissues	Toxicity assay	Reference
Alginate	polyethylene glycol dimaleimide	^{1}H NMR (Solvent: D_2O), FTIR, Small angle X-ray scattering (SAXS)	Nil	Dynamic viscosity of polymer -mucin using Rheometer & tensile study using tensile machine	Tablet	Porcine intestine	Cell viablity (Normal human dermal fibroblast -NHDF cells)	[104]
Chitosan	6-maleimidohexanoic acid	^{1}H NMR (Solvent: D_2O & DMSO-d6), FTIR	Indirect Ellman's assay	Tensile test using texture analyser	Tablet	Porcine cheek pouch tissue	MTT test (Human gingival fibroblast cells - HGF)	[95]
PLGA-PEG	Methoxypolyethylene glycol maleimide	TEM, Zetasizer & Small-angle neutron	Encapsulation efficiency (EE%) and	Retention test	NP	Lamb urinary bladder mucosa	Slug mucosal irritation study	[105]

		scattering (SANS)	loading capacity (LC%)					
HEC 720 kDa	N-(4-bromophenyl) maleimide	^{1}H NMR (Solvent: D_2O), FTIR	^{1}H NMR and elemental analysis	Tensile test using texture analyser	Spray coated tablet	Sheep buccal tissue	Cell viability (Caco-2) and planaria toxicity and fluorescence intensity test	[106]

Recently, the "click chemistry" of synthesised vinyl sulfone was demonstrated using the divinyl sulfone by reaction with a hydroxyl group. The degree of modification can be changed by optimizing three parameters such as (i) pH (which determines the concentration of the alkoxide ion [O−]), (ii) reaction time, and (iii) DVS to OH molar ratio [107]. When the DVS/OH ratio and pH are high (pH 9 and 10), the degree of substitution of vinyl sulfones is higher [107].

However, the synthesis of DVS by click chemistry may result in a crosslinked hydrogel that is unsuitable for mucoadhesion. Due to the weak cohesive force in the semisolid hydrogel itself and further dilution with body fluid, the dosage form is a weak site for mucoadhesion. Therefore, a solid dosage form is preferred as it could provide a better adhesion site as it is stronger than the mucus layer [108].

2.4.1.5.Catechol

Catechol was discovered in mussels and is categorised as a polyphenolic material as it has many phenolic groups in its molecular structure [109]. Chemical modification of polymers with this phenolic compound showed better molecular adhesion properties due to various chemical interactions with the surface via electrostatic interactions, π–π stacking, and hydrogen bonding [109]. The foot proteins of mussels contain (3,4-dihydroxyphenylethylamine or DOPA), an adhesive protein which is the key to increase the wet-resistant adhesion. Depending on DOPA level, DOPA can form reversible non-covalent or irreversible covalent bonds with chemical and inorganic substrates [110].

This binding leading to adhesion depends on the oxidation of the catechol units of the DOPA groups. High DOPA oxidation results in interface failure but high cohesive strength, whereas low DOPA oxidation results in better adhesion but lower cohesive strength [111]. In addition to the level of DOPA, pH is also a factor affecting DOPA adhesion [112].

There are a few studies on the synthesis of DOPA-modified cellulose, such as with NaCMC, BC and CNC [113–115]. DOPA has the unique property of enhancing the adhesion effect under humid conditions; therefore, the modification of synthesised polymers with DOPA targets transdermal delivery dosage forms such as films and patches. Other attempts of developing mucoadhesive polymers by catechol conjugation, were reported with chitosan, PEG, and PVA, where the conjugated polymers with catechol shows a high adhesion time and mechanical strength to buccal tissue [116,117].

2.4.1.6.Tannic acid derivatives (Pyrogallol)

Tannin is a plant polyphenol used to form resins in the manufacture of adhesives due to its chemical structure and reactivity with formaldehyde [118]. It is subsequently gaining renewed interest for applications in the food, leather and medical industries [119].

Pyrogallol is derived from hydrolysed tannin/ tannic acid, by treating tannic acid with acid and catalyst, followed by thermal decarboxylation with catalyst [120]. It can form non-covalent bonds such as hydrogen bonds and has a high affinity for proteins, peptides, DNA/RNA, and polysaccharides [121]. In a study by Choe et al., it was shown that the pyrogallol group is better for polymer-metal adhesion than catechol [122]. In a mucoadhesion study of synthesised pyrogallol conjugated with PEG; it was found that adherence to the mucous layers of the oesophagus has lasted for less than 8 hours. This result was better than the polymer with catechol groups [123].

2.4.2. Blending

Polymer blending is a process in which two or more polymers are combined, resulting in better, improved, or tailored properties and functions [124,125]. This method of polymer modification can lead to significant benefits in applications such as effective drug delivery. Polymer blending has attracted considerable interest as it is a fast and cost-effective way to produce versatile polymeric materials [124]. In contrast, novel and new polymers can take years to develop and commercialise and are expensive.

However, the challenge with polymer blends is that the physical properties of the blended polymers depend on the properties of the individual homopolymers and intramolecular interactions. Formulators need to understand and investigate the miscibility and compatibility of the different blends. In the manufacture of pharmaceutical preparations, cellulose and its derivatives (ethers and esters) are among the commonly used excipients. Therefore, most polymer blends for mucoadhesive formulations used the most common excipients on the market. According to Bakhrushina et al, the most commonly used polymers for mucoadhesive dosage form formulations are cellulose derivatives, sodium alginates, xanthan gums, carbomers and PEGs [65].

Table 2.6 shows a list of some pharmaceutical formulations in which different polymers were mixed to improve the mucoadhesive properties of the dosage form. Although all the papers describe the use of at least one cellulosic polymer, a comparison is not possible due to differences in the quality of the polymer. Most of the polymer blends in Table 2.6 use the.

Table 2.6. Blending of polymers with cellulose and cellulose derivatives to improve the mucoadhesive properties

Polymers	Dosage form	Techniques	Characterisation methods	Mucoadhesion method	Biological tissues/ animal	Note	Reference
Chitosan & HEC	Film	Solution casting	FTIR, XRD, SEM, TGA, Swelling test, Mechanical test,	Tensile test using texture analyser	Porcine buccal tissue	• Film more elastic with HEC • Adhesion of chitosan film decrease with addition of HEC	[126]
Poly (acrylic acid) (PAA) & (hydroxypropyl) cellulose (HPC))	Film	Cross-linking of these materials with γ-radiation	Turbidity test, Viscometric test, Luminescence Spectroscopy, SEM, Swelling test	Rotating disk method	Porcine buccal tissue	• Film more elastic with HEC • Cross-linked PAA-HPC blends films with >70 mol % HPC improves adhesion similar to pure PAA films	[127]
PEO + CMC, PEO + PAA and PEO + alginate	Powder	Fiber spinning	FTIR & SEM	Adhesive test using texture analyser	Nil	• The blend containing PEO and CMC appears to offer the best mucoadhesive	[128]
PAA + MC	Film	Solution casting	DLS, SEM, Turbidity test	*In vivo* retention study with rabbit cornea	Chinchilla rabbits	• PAA + MC blends and cast at 3.4<pH<4.5 form uniform & transparent film	[129]

					(2.5–3.0 kg	and show a complete miscibility. • PAA + MC films exhibited a good adhesive and retention time within 30–60 min	
Carbopol + poloxamer + HPMC	Film	Solution casting	FTIR, Dynamic mechanical thermal analysis (DMTA), swelling ratio	Adhesive force and tensile strength using texture analyser with plastic plate & bioadhesive force with biological tissue	Fundus of rabbit rectum	• The best ratio for the Carbopol/poloxamer/HPMC mucoadhesive polymeric film in terms of flexibility, comfort, long residence time, swelling, and bioadhesive force was 35/30/35 (wt/wt/wt).	[130]
Cellulose nanofibrils (CNFs) + Tannic acid (TA) + PEI	Film	Solution casting	FTIR, XRD, SEM, water absorption test	Lap shear tensile tests with PET Film using Universal testing machine	Nil	• The new adhesive film can be used in multiple surfaces. • The shear strength of adhesive film is 392.2 ± 32.2 kPa, the wet shear strength is 144.7 ± 20.1	[115]

						kPa, and the water absorption rate is 12.8 ± 5.9%.	

physical mixing method. There are two other basic methods when mixing polymers, namely the core-shell model and the block-copolymer model [131].

2.4.3. Cationic cellulose

Cationic functionalisation of cellulose is widely used, especially in the paper industry [132], cosmetics [133], textiles, flotation and flocculation, and drilling fluids. Electrostatic interactions between the positively charged polymer and anionic mucus glycoproteins are one of the non-specific mucoadhesion mechanisms as illustrated in Figure .2.2. Cationic polymers have positive charges that enhance their mucoadhesive properties while maintaining their polymer properties.

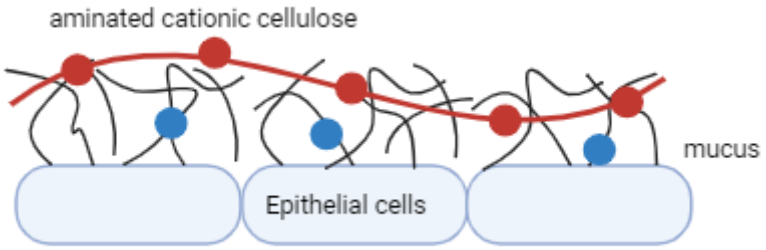

Figure 2.3: Illustrated the positively charged amino acids interact electrostatically with negatively charged mucus glycoprotein substructures like sialic and sulfonic acid substructures [134]. Created with BioRender.com.

A modified cationic polymer is the result of a common quaternisation process using a quaternary ammonium monomer, such as 2,3-epoxypropyltrimethylammonium chloride (EPTMAC) [132], glycidyl trimethyl ammonium chloride (GTAC)[133], trimethyl ammonium, and dimethyldodecyl ammonium [133].

Modification of cellulose as a cationic polymer changed the polymer properties. For example, modified cationic HEC was used to increase the hydrophilicity of the polymer [133]. This property is essential for an excellent mucoadhesive polymer [135]. In a study by Hansen and colleagues, they discovered that 0.1% cationic-HEC increased the permeability of drugs through the nasal epithelium. It is suggested that the mucoadhesion of the polymer and a change in the gating of the tight junctions may be a possible explanation for the mechanism of action. [136]. The dissociation part of the cationic modified HEC is positively charged, which attracts negatively charged mucin via an electrostatic reaction and exhibits significant mucoadhesive properties [137].

Another method to obtain cationic cellulose is the insertion of primary amine groups by reductive amination of oxidatively cleaved cellulose [138]. The modified cellulose aminocellulose has been shown to have better mucoadhesive properties, both in terms of total adhesion work and maximum detachment force (measured with a texture analyser) than unmodified cellulose. It is also more soluble in water compared to quaternised cellulose derivatives.

2.4.4. Thiolated cellulose

Thiolated polymers (thiomers) are modified polymers with free thiol groups. Through thiol/disulfide exchange processes, thiomers can form covalent bonds with cysteine-rich subdomains of mucus glycoproteins [139].

In general, the reactivity of thiomers is strongly influenced by the pKa value of the thiol group of the chosen ligand. The lower the pKa value, the more thiolate anions (the reactive form of sulfhydryl) are available at physiological pH. Thiomers are susceptible to thiol oxidation at pH above 5 in aqueous solution unless they are sealed under inert conditions [140,141].

Two different methods can be used to modify cellulose as thiomers. Cationic thiolated cellulose can be synthesised by reacting the 2-amino position of glucosamines in the polymer chain. For example, in the HEC-cysteamine reaction, HEC is reductively aminated by reacting the aldehyde groups with cysteamine at pH 5 to form a primary amine [142,143]. HEC-cysteamine exhibits stronger basicity than the primary amine groups on the chitosan backbone by adding a stable secondary amine [142].

In anionic thiomers, the conjugation of sulfhydryl compounds to the backbone of cellulose is mediated by carbodiimide coupling between amines and carboxylic acids [144,145]. An example of this is carboxymethylcellulose-cysteine (CMC-Cys), where the optimal pH value for the coupling reaction of the sulfhydryl group to the NaCMC polymer is 6 [146]. The modified CMC-Cys improves permeation by breaking the tight intercellular linkage.

2.5. Other cellulose sources as potential mucoadhesive polymers

2.5.1. Bacterial cellulose

Bacterial cellulose or nata de coco is sold as a food product in the market, primarily in Asia. Bacterial cellulose is similar in composition to cellulose, an insoluble, dietary fibre. It is commercially produced via fermentation of sugar water by *Acetobacter* sp. bacteria. In Asian countries, nata de coco is served as a dessert and sweet drink.

As an exopolysaccharide polymer, bacterial cellulose is synthesised and secreted by microorganisms outside the cells [147]. Using various techniques and methods, bacteria belonging to the genera of *Acetobacter, Rhizobium, Agrobacterium,* and *Sarcina* can produce bacterial cellulose, with the genus *Komagataeibacter* sp. (formerly known as *Gluconacetobacter* sp.) being the most efficient producer [148–151]. Bacterial cellulose was discovered in 1886 and the fermented product was described as a 'vinegar plant' that developed a translucent, jelly-like mass upon cultivation [149]. The quality of bacterial cellulose is superior to wood-derived nanocellulose [9]. This can be attributed to the high purity of BC.

Bacterial cellulose costs about 800–1200 USD/ton in the market, primarily due to. culture media accounting for about 30% of the total cost [152]. Thus, bacterial cellulose is mainly produced via agricultural residues such as inedible coconut water from old coconuts, pineapple wastewater juice [153,154], sugar cane juice [154], wastewater from candied jujube processing plant [155], rotten fruits[156], dry olive mill waste [157], palm date fruits, fig fruits, sugarcane molasses [158], sugar beet molasses, and cheese whey [159] as a carbon source.

2.5.1.1.Physical, chemical and mechanical properties

Bacterial cellulose has a similar molecular formula $(C_6H_{10}O_5)_n$ to plant cellulose, but these materials differ in their physicochemical properties. In wood or plant-based cellulose, the cellulose chains are layered as nanofibrils, bound together by a matrix composed of hemicellulose and lignin. Cotton fibre is plant cellulose but contains negligible amounts of lignin and hemicellulose. Similar to plant cellulose, bacterial cellulose is also arranged in polysaccharide chains, but without hemicellulose and lignin as part of the cell wall [160].

Structurally and mechanically, bacterial cellulose varies and is strongly influenced by cultivation systems. According to Chawla et al., the culture system affects the degree of crystallinity, crystallite size and Iα cellulose content of bacterial cellulose [148]. Bacterial cellulose consists of a random network of microfibrils. These microfibrils are arranged in a web-like pattern and contribute to the higher tensile strength of cellulose. Although wood, plant and bacterial based cellulose have the same chemical building elements, their structural organisation distinguishes their mechanical properties [161].

The mechanical strength of bacterial cellulose was predicted based on the stiffness of a single fibril (35 – 90 nm) by the young modulus with a value of 78 ± 17 GPa using atomic force microscopy. Compared to the theoretical study of the mechanical strength of cellulose (130-170 GPa), the value of the single bacterial cellulose fibril is high because cellulose is arranged in fibre bundles [162].

The macromolecules in cellulose are arranged in parallel to form cellulose microfibrils which are connected by hydrogen bonds and van der Waals forces [163]. Within these cellulose fibrils, there is a combination of highly ordered (crystalline) structures and disordered (amorphous) structures [164]. Crystallinity, which measures structural order, is the volume ratio of crystalline to crystalline plus amorphous content. The crystalline structure is important because the physicochemical properties of all celluloses depend on the degree of crystallinity [165]. Crystallinity is over 90% for plant-based fibres and 60-70% for wood-based fibres [166]. However, for cellulose biosynthesised from bacteria, the degree of crystallinity is higher (above 90%) and the formation of microfibrils depends on the bacterial strain and culture conditions [167,168]. Bacterial cellulose also has a higher degree of polymerization (DP) than plant cellulose. Bacterial cellulose produced by *Acetobacter xylinum* has a DP of 16,000 compared to cotton, (3,000-14,000), and wood cellulose (7,000-10,000 [169].

2.5.1.2.Bacterial cellulose for mucoadhesion

Mucoadhesion of bacterial cellulose can be achieved by modifying the polymer. In a study by Naveed et al., it was found that a synthesised bacterial cellulose -G-poly(acrylic acid) hydrogel disc exerted better mucoadhesion, which was attributed to the carboxyl group of the acrylic acid of the hydrogel and the mucus in the intestinal tissue [170]. Cross-linking between anionic poly (acrylic acid) and bacterial cellulose by electron beam creates an interpolymer network, resulting in a strong gel. However, the exact amount of polyacrylic acid grafted onto the bacterial cellulose in the formulation containing 30% PAA and 70% bacterial cellulose, which exhibits the highest mucoadhesion property, cannot be quantified. The mucoadhesion study was performed by adhesive test on goat intestinal tissue using a texture analyser.

There are several other strategies to overcome the mucosal lining of the body tissue which include mucopenetration and mucolytic agent, which we will not discuss further in this chapter. This includes the conjugation of bacterial cellulose with mucus-penetrating agents such as cell-penetrating peptides (CPPs) [171] and mucolytic agents such as the pineapple-derived enzyme bromelain [172]. Bacterial cellulose has been shown in many studies to be a preferred substitute for biomedical materials due to its biocompatibility and high mechanical stability.

2.5.2. Cellulose Nanocrystal (CNC)

2.5.2.1.Physical, chemical and mechanical properties

The definition of cellulose nanocrystals is quite vague as it is used interchangeably to describe all types of nanosized cellulose substrates, including CNCs, cellulose nanofibrils (CNFs), and

bacterial cellulose. However, the definition of nanomaterials from sizing is a geometry with at least one dimension smaller than 100 nm [173]. Nanocellulose can be classified as either CNFs or CNCs. Both are derived from different biological sources and differ in their physical and chemical properties. In terms of morphology, CNCs are referred to as nanowhiskers due to the smaller size of the nanocrystals of 10-20 nm, while CNFs are easily recognised by a long-fibrillar network [174]. The differences in morphology of the two nanocelluloses are due to the different extraction procedures. CNFs are isolated by mechanical treatments, in contrast to CNCs, which are largely isolated by acid hydrolysis [175].

The nanowhisker characteristic of CNCs is not an essential description of the physical characterisation of CNCs. The main physical dimensions and size depend on the cellulose source or hydrolysis conditions (acid type, reaction time and temperature). The size of CNCs is measured by diameter (D), length (L), and aspect ratio (L/D). The length of the CNCs varies on average from 200 nm to 600 nm. The width of the CNCs can vary from 3 nm to 50 nm. There are also cases where the diameters are large due to aggregation of materials [176].

CNCs are usually made from wood pulp. However, due to the high cost of these raw materials, an alternative is becoming more interesting: the use of residues from agroforestry and other cellulose sources. Wood pulp is in high demand in other industries, such as the paper and furniture industries. According to reports, the cost of producing CNCs using wood pulp ranges from 3632 to 4420 USD/tonne of CNCs (dry equivalent). The main cost of manufacturing CNCs using cellulose is 38% to 45% of the total manufacturing cost, which is about 763 USD/tonne in 2019 [177].

For better functionalisation of cellulose nanocrystals (CNCs), these polymers need to be modified, which changes their physical, chemical, and biological properties. These specific properties include an increase in hydrophilicity, better thermal stability, flexibility, and rigidity of the polymers [178]. There are numerous processes for altering or modifying cellulose, such as grafting, cross-linking, blending and the formation of composites.

The main challenge in the chemical functionalisation of CNCs is to change the surface of the polymer while maintaining the original morphology and crystal content [179]. CNCs are not soluble in water or most organic solvents. Due to the strong hydrogen bonding effect caused by the drying process, the hydroxyl group of CNCs tends to aggregate irreversibly [15].

Challenges working with this natural polymer are the degradation and batch-to-batch variation. Another aspect to be considered in the surface chemistry of CNCs is the surface charges, generated by negative sulfate esters during sulfuric acid hydrolysis by condensation esterification (sulfation) between the surface hydroxyl groups and H_2SO_4 [14]. Therefore, the

CNCs are strongly negatively charged and well dispersed as an aqueous colloidal suspension. An additional challenge is that nanocellulose is a polar polymer, so working with a hydrophobic polymer presents a dispersion problem [180].

2.5.2.2.CNC for mucoadhesion

There are few studies on the mucoadhesive properties of CNCs. A comparison between different types of nanocellulose showed that Tempo-CNF exhibited the highest adhesion strength under gastric conditions, while CNFs and CNCs showed intermediate bioadhesion with mucin using a flow-through retention method [181]. This technique is performed to monitor the retention of mucoadhesive polymers after the mucosal tissue has been washed with a biological flow.. The modified Tempo CNFs exhibited a high carboxylate content; allowing them to be easily dispersed as a colloidal suspension. It was suggested that the carboxyl groups in Tempo-s form hydrogen bonds with mucin glycoproteins.

Interestingly, CNC carries negative charges and showed intermediate adhesion with mucus tissue and mucin solution in simulated intestinal fluid. The studies also confirmed that CNC particles could not penetrate the intestine [182]. It was also agreed that CNC should be excreted as indigestible [183]. CNC was also used to enhance formulations with different mucoadhesive polymers by blending and grafting [184,185]. In a hydrogel formulation, CNC provided excellent mechanical properties and a large adsorption surface [186].

2.6. Conclusions

Cellulose and cellulose derivatives serve as excipients in a variety of delivery systems. Cellulose has inherently poor mucoadhesive properties. To improve the mucoadhesive properties of cellulose, various strategies are used such as modification with groups that specifically bind to mucosal tissue, modification into a charged polymer, thiolation of polymers, and blending with other mucoadhesive polymers. In this chapter, we have discussed the necessary understanding of these existing techniques and innovative systems for the use of cellulose and cellulose derivatives as excipients in mucosal drug delivery.

In summary, work on cellulose and cellulose derivatives using these strategies is still limited. The reason could be that it is difficult to achieve uniform modification, suitable polymer ratios and the desired degree of modification for effective mucoadhesion time. Although the above strategies improve the mucoadhesive properties of cellulose and cellulose derivatives, additional modifications or incorporation of other adhesion-promoting components such as mucopenetrator could provide more adhesive strength for effective

mucoadhesion. It is also worth investigating other cellulose types such as BC and CNC as potential mucoadhesive polymers for future work.

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Chapter 3.

Synthesis of methacryloylated hydroxyethyl cellulose and development of mucoadhesive wafers for buccal drug delivery

(QUAD system contribution of Fhataheya Buang: 60% of conception and design, 100% of data collection, 70% of data analysis and conclusions, and 70% of manuscript preparation).

Chapter 3.

Synthesis of methacryloylated hydroxyethyl cellulose and development of mucoadhesive wafers for buccal drug delivery

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Abstract

Non-ionic hydroxyethyl cellulose (HEC) has limited mucoadhesive properties for application in transmucosal drug delivery. In this study, HEC was chemically modified by reaction with glycidyl methacrylate. This allowed the introduction of methacryloyl groups to HEC structure to make it capable of forming covalent bonds with the sulfhydryl groups present in the mucin glycoprotein to achieve enhanced mucoadhesive properties. The results showed a successful modification of HEC as confirmed by ^{1}H NMR and FTIR spectroscopies. The quantification of methacryloyl moieties was conducted using HPLC. The toxicity studies using *in vivo* planaria acute toxicity assay, *in vivo* planaria fluorescent test, and *in vitro* MTT assay with Caco-2 cell line confirmed that the chemical modification of HEC does not result in any toxicological effects. Mucoadhesive wafers were developed based on parent and modified HEC as a model dosage form for buccal delivery. The mucoadhesive properties of modified HEC assessed using a tensile test were found to be significantly better compared to unmodified HEC.

Keywords: hydroxyethyl cellulose; mucoadhesion; methacryloyl; transmucosal delivery; wafers

3.1. Introduction

The delivery of drugs through mucosal membranes lining the body is a non-invasive option for achieving local and systemic effects. Transmucosal drug delivery offers advantages such as increased drug residence time, improved bioavailability, and avoidance of the first-pass effect or pre-systemic metabolism [1,2,3]. Oromucosal, gastrointestinal, ocular, vaginal, intravesical, nasal, and rectal routes are among the established routes of transmucosal drug delivery. In any of the mentioned routes, poor drug retention on the site of action is usually an issue. Thus, to increase the drug residence on the mucosa, mucoadhesive materials are commonly used in the formulations as they facilitate dosage form adhesion to the tissues [4].

Cellulose and its derivatives are biocompatible, renewable, and non-toxic polysaccharides. They belong to the first generation of mucoadhesive polymers as they may interact with mucosal surfaces via physical attraction forces such as hydrogen bonding [5,6]. Compared to cationic and anionic polymers, the non-ionic hydroxyethyl cellulose (HEC) exhibits limited mucoadhesive characteristics [7,8].

Blending HEC with other mucoadhesive polymers is one of the methods used for enhancing mucoadhesive properties of dosage forms. However, the dosage form's adhesiveness may potentially be impacted by the interpolymer complexation between HEC and other polymers [9]. Therefore, alternative strategy to enhance mucoadhesive properties of HEC is through its chemical modification to introduce adhesive groups. For example, modification of HEC with cationic and thiol groups has been reported previously by other researchers [10,11]. Previously our research group demonstrated that introduction of methacryloyl groups into chitosan [12], gellan gum [13] and poly(2-ethyl-2-oxazoline) [14] leads to a substantial enhancement in the mucoadhesive properties of these polymers. This modification was achieved by reacting chitosan, gellan gum and ethylene imine-co-2-ethyl-2-oxazoline with methacrylic anhydride. The enhancement in mucoadhesive properties is due to the ability of methacryloyl groups to form covalent bonds with thiol groups present in mucin under physiological conditions.

In this study, we have modified non-ionic HEC by reaction with glycidyl methacrylate as a new strategy to introduce mucoadhesion-enhancing groups into polymers. The resulting derivatives were characterised using ^{1}H NMR and FTIR spectroscopies as well as hydrolysis with subsequent quantification of methacrylic acid with HPLC. The toxicological properties of these new HEC derivatives were evaluated using acute toxicity and fluorescence assays in planaria as well as MTT cytotoxicity assay in Caco-2 cells. The parent as well as the modified polymers were subsequently formulated into the wafers as a model dosage form for buccal drug

delivery. The porosity, mechanical and mucoadhesive properties of these wafers were evaluated.

3.2. Materials and Methods

3.2.1. Materials

HEC (720 kDa), triethylamine (TEA), tributyl ammonium bromide (TAB), glycidyl methacrylate (GMA), hydrochloric acid, benzalkonium chloride, sulfuric acid, methacrylic acid and sodium hydroxide were purchased from Sigma Aldrich Co., Ltd., Gillingham, UK. N, N-dimethylformamide (DMF) was supplied by SLS Supplies Ltd., Nottingham, UK.

Cell culture materials DMEM High Glucose (Capricorn Scientific GMbH, Germany), foetal calf serum (GE Healthcare Life Sciences, Chicago, IL, USA), penicillin/streptomycin (Nacalai Tesque Inc., Kyoto, Japan), CellTiter 96 Aqueous MTS reagent powder (Promega Corporation, Wisconsin, USA) were used for cell viability assay. The Caco-2 cells were received from Dr Sharifah Aminah, Faculty of Pharmacy, in UiTM Puncak Alam, Malaysia.

The freshly excised sheep's upper and lower lips were sourced from PC Turner Abattoir (Farnborough, Hampshire, UK).

3.2.2. Modification of HEC

1% (w/v) solution of HEC was prepared by dissolving HEC in 0.1 M NaOH. Then TEA was added to the HEC solution as a catalyst. GMA was added to these solution mixtures at different molar ratios, as shown in Table 3.1, and constantly stirred at 25 °C for 24 hours. The reaction products were purified using dialysis via membranes with molecular weight cutoff of 12–14 kDa. Deionised water was changed 8 times (4.5 L) a day for over 48 h during dialysis. The final products were subsequently freeze-dried.

Table 3.1. Details on HECGMA synthesis.

ID	Molar Ratio [HEC]:[GMA]	GMA (μL)	TEA (μL)
HECGMA low	[1]:[1]	225	240
HECGMA medium	[1]:[3]	675	240
HECGMA high	[1]:[6]	1350	240

3.2.3. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

Polymer solutions (20 mg/mL) were prepared in D_2O in NMR tubes of 5 mm diameter. The ^{1}H NMR spectra were recorded using a 400 MHz Ultrashield PlusTM B-ACS 60 spectrometer (Bruker UK Ltd., Coventry, UK) and were analysed using MestReNova (Mnova) Version 6.0.2-5475 (Mestrelab Research, S.L., Spain).

3.2.4. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of freeze-dried samples were recorded using Spectrum 100 FTIR Spectrophotometer (Perkin–Elmer UK Ltd., Buckinghampshire, UK) with scanning from 4000 to 650 cm^{-1} at 4 cm^{-1} resolution, and accumulation of 16 scans. The data were analysed using a six-scan average per sample generated by Spectrum One software (Perkin–Elmer UK Ltd., Buckinghampshire, UK).

3.2.5. High-performance liquid chromatography (HPLC)

For the analysis of methacryloyl groups content, 40 mg of polymer samples were dissolved in 8 mL of 0.01 M sulfuric acid and solutions were refluxed for 4 hours at 50°C until a complete degradation of the samples. Methacrylic acid formed as a result of this reaction was quantified using HPLC.

The HPLC procedure for the analysis of methacrylic acid was adapted from Paleologos and Kontaminas, 2005 and was carried out on an Agilent Infinity 1200 HPLC system with an Aminex 87H (Biorad, Watford, UK) column at 40°C [15]. Isocratic elution was applied at 0.6 $mL \cdot min^{-1}$ with 0.01 M sulfuric acid solution and methacrylic acid detection was performed in a diode array detector (Agilent Infinity 1200 Series, Didcot, UK) at 200 nm wavelength.

Methacrylic acid was dissolved in 0.01 M sulfuric acid to form the standard stock solution, which was diluted with deionised water to form standard solutions with concentrations ranging from 0.1 to 59.0 µmol/mL (see Appendix I), used for the generation of external calibration curve and methacrylic acid quantification in the samples.

3.2.6. Planarian acute toxicity assay

Schmidtea mediterranea planaria were provided by Oxford Brookes University and were kept in artificial pond water (APW: 5 M NaCl, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M $MgCl_2$ and 1 M KCl were dissolved in 50 mL ultra-pure water (UPW).and further diluted in 20 L of UPW) at room temperature. Planaria were given chicken liver once a week, and the APW was changed every week following their feeding. Planaria (1.0–1.5 cm long) were placed each in 24 wells of a

plate culture using a slightly modified version of the procedure [16,17]. Briefly, 1 mL of HEC and HECGMA solutions at various concentrations (0.05% w/v, 0.10% w/v, 0.25% w/v, 0.50% w/v and 1.00% w/v) were added into each well. A solution of 1% w/v benzalkonium chloride (BAC) in APW was used as a positive control that typically causes severe irritation of mucosal membranes [18]. All test materials were dissolved in APW. The plates were stored at room temperature in the dark. The number of living and dead planaria was determined after 24, 48, and 72 h of the acute toxicity test. Planaria that did not move after a gentle agitation were considered dead.

3.2.7. Planarian toxicity fluorescent assay

Following the experiments on acute toxicity assay, where the worms were exposed to 1% (w/v) polymer solutions for 24 hours, these planaria were subsequently exposed to 0.1% (w/v) sodium fluorescein solution in APW for 1 min. The worms were then washed in APW for 15 min to remove residual dye. In order to immobilise the planaria, a glass slide containing the worms was covered with a few drops of a 2.0% (w/v) agarose solution and placed on a flat surface of ice flakes (−0.5 to −0.8 °C) until the gel solidified. Leica MZ10F stereomicroscope (Leica Microsystems Ltd., Wholesaler, UK) equipped with DFC3000G digital camera at 2.0× magnification, 160 ms exposure duration, and gamma 0.7 were used to record fluorescence images of the worms. Permeation of sodium fluorescein into the worms was evaluated using ImageJ software (version 1.8.0_112) as described in Shah et al. [16]. The acquired mean value was normalised by dividing the fluorescence intensity by the total area (in cm^2) of each planaria.

3.2.8. *In Vitro* cytotoxicity of polymers

The cytotoxicity of each polymer was evaluated using Caco-2 cells. The cells were grown in DMEM High Glucose fortified with 10% foetal calf serum and 1% penicillin/streptomycin. It was kept at 37 °C in an incubator with 5% CO_2 and 100% relative humidity.

At a density of 1×10^4 cells per well, cells were seeded in 96-well plates and incubated for an overnight period at 37 °C in humidified air containing 5% CO_2 to promote cell attachment. The cells were then treated with various concentrations of the polymers (1%, 0.5%, 0.25%, 0.1% and 0.05% w/v) for 24 hours. The negative control group consisted of untreated cells and was considered as 100% of viable cells. The media were changed with fresh growth medium following the end of every treatment. Each well received 20 µL of 5 mg/mL MTT solution (in the dark). The cells were further incubated for 4 h at 37 °C in a humidified 5% CO_2 incubator.

100 µL of DMSO was added, mixed thoroughly and incubated for 10 min. The absorbance was measured at 540 nm with Infinite 200 PRO microplate reader (Tecan Group Ltd., Maennedorf, Switzerland).

3.2.9. Preparation of wafers

The wafers were prepared from 1% (w/v) solutions of HEC and its derivatives in deionised water. 1.5 g of HEC, and HECGMA solutions were poured into each well in 24 well plates. The plate was covered with holed aluminium foil and was left under a fume hood for an hour. It was then frozen in a freezer at −20 °C overnight. The wafers were prepared by freeze-drying in a Heto Power Dry LL3000 Freeze Dryer (Thermo Scientific UK Ltd., Leicestershire, UK) over 48 h. The wafers were placed in sealed containers and stored in a fridge at 4 °C.

3.2.10. Physical characterisation of wafers

Wafers were examined for physical features (colour and texture). A digital microbalance was used to weigh the wafers, and their average weight ± standard deviations were calculated. The wafers were each measured for thickness using an electronic Vernier calliper, and the average thickness ± standard deviations were calculated. SEM analysis of the wafers provided more information on their porous structure. The wafers were mounted on an aluminium stud and secured with double-sided carbon tape adhesive. SEM images were generated using FEI Quanta 600 FEG (FEI Company, Czech Republic).

3.2.11. *Ex Vivo* mucoadhesion study of wafers

The method was slightly modified from several studies [19-21]. A TA-XT Plus Texture Analyser (Stable Micro Systems Ltd., Surrey, UK) with a 5 kg load cell was used to study the mucoadhesive properties of all the formulations. Sheep buccal tissue was cut into squares and secured onto mucoadhesion rig with a 20 mm opening. Upon testing, the device and tissues were immersed in a 37 °C water bath.

The wafers were attached to the 12 mm diameter aluminium probe with sticky adhesive tape and lowered to contact the mucosa. The following test parameters were used: pre-speed test 0.5 mm/s; test speed 0.5 mm/s; post-speed test 1.0 mm/s; applied force 0.5 N; contact time 60 s; trigger type auto; trigger force auto; and return distance 20 mm.

3.2.12. Statistical analysis

SPSS (version 17) was used to perform a two-tailed student t-test as a statistical tool with p values < 0.05 considered statistically significant.

3.3. Results and discussion

3.3.1. Synthesis of methacryloylated HEC

It can be expected that the reaction of GMA with HEC leads to formation of methacryloylated derivatives (see Figure 3.1), which is similar to the reactions of this reagent with other hydroxyl-containing polymers reported in the literature [22,23]. In general there are two reaction routes possible with the use of GMA in chemical modification, via transesterification and epoxide ring opening mechanisms [22-26]. We conducted the synthesis in alkaline protic solvent (containing NaOH and TEA as bases) which resulted in the reaction favouring epoxide ring opening than transesterification as reported by Fajardo et al. and Reis et al. [22,27]. The structure of the resulting derivatives of HEC was evaluated using ^{1}H NMR spectroscopy (see Figure 3.2).

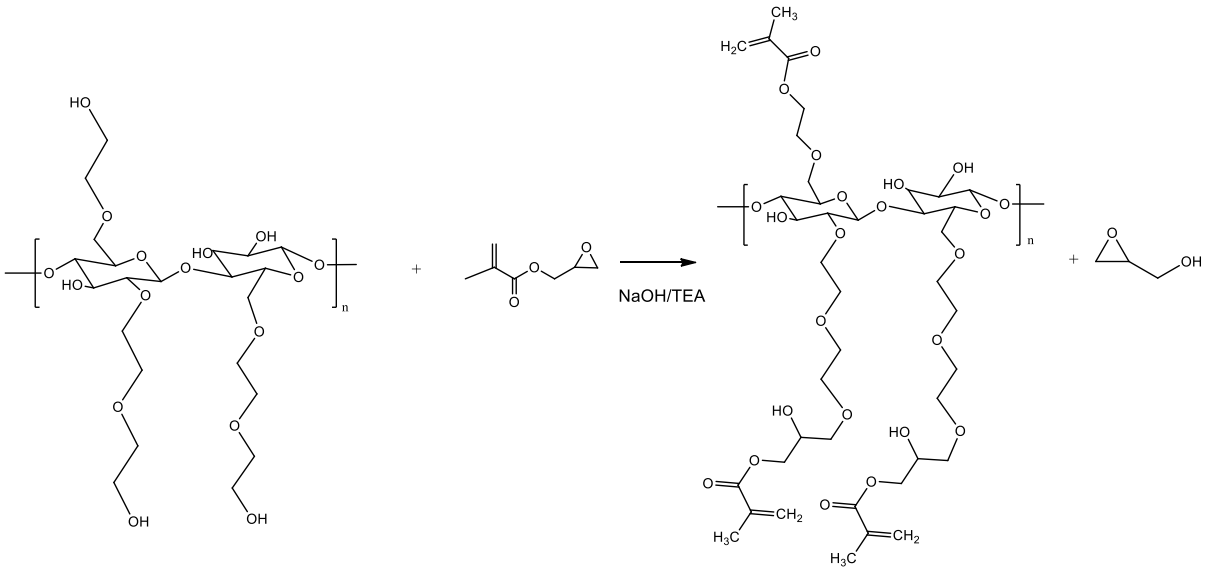


Figure 3.1. Proposed reaction scheme of HEC with glycidyl methacrylate at alkaline conditions (pH 13.3).

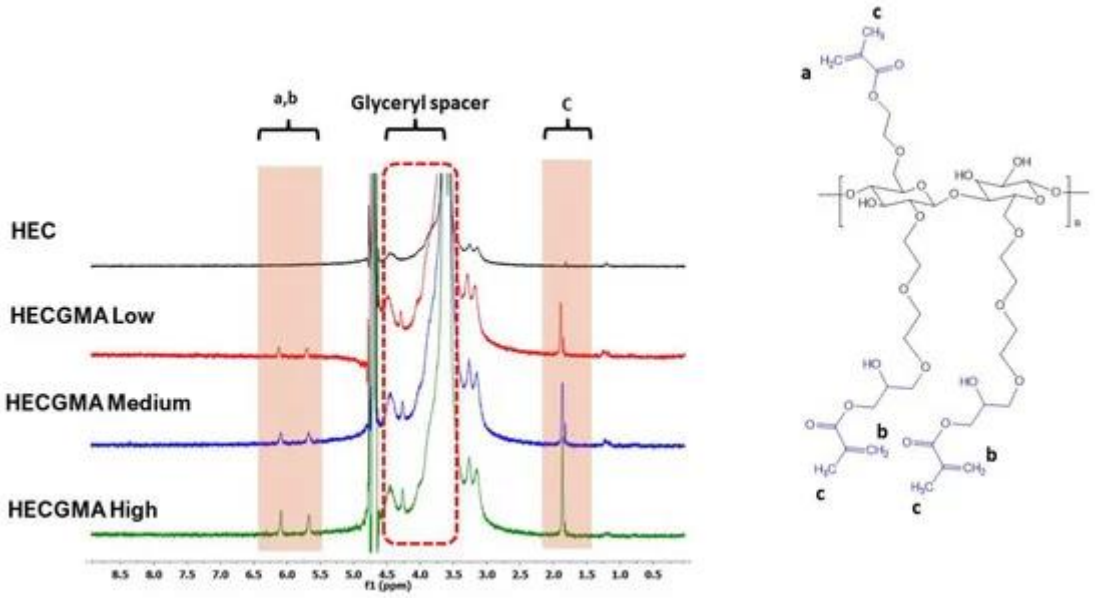
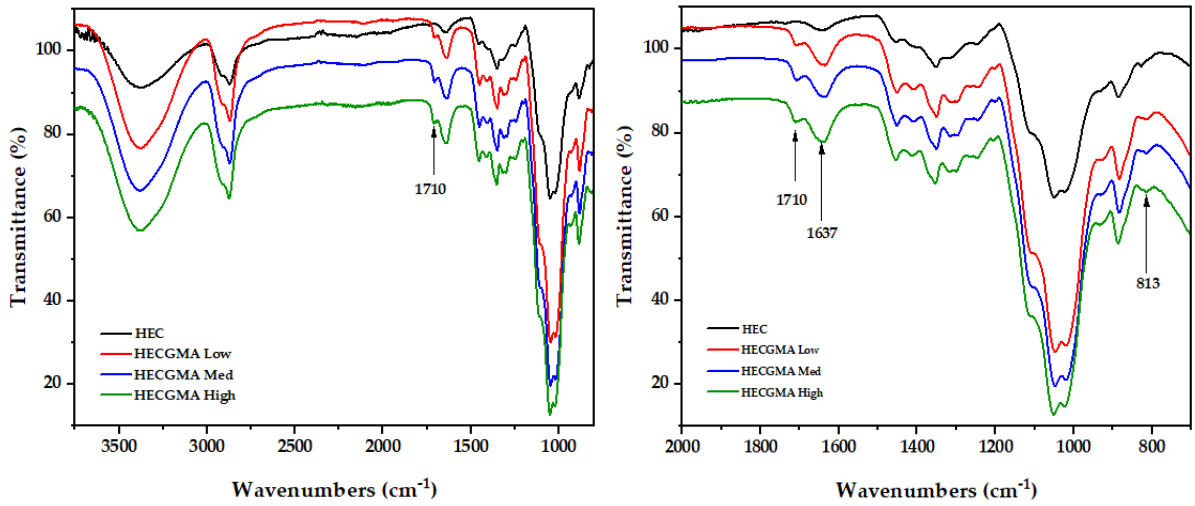


Figure 3.2. Structure and ^{1}H NMR spectra of unmodified HEC and HECGMA prepared at various molar ratios of HEC to GMA.

The ^{1}H NMR spectra of modified HEC show the signals at 5.69 and 6.10 ppm, which correspond to the protons of methacryloyl groups [12,13]. The signals that appeared in the spectra of methacryloylated HEC at 1.89 ppm correspond to protons of methyl groups from methacryloylation [13]. The peaks at 1.22 and 1.82 ppm belong to unidentified structures within HEC, which was similarly found and reported by Ray et al. [28].

Unfortunately, the extent of HEC methacryloylation cannot be evaluated accurately using the analysis of ^{1}H NMR spectra. HEC has a complex structure similar to other heteropolysaccharides that generate broad signals in the ^{1}H NMR spectra, which overlap with glyceryl spacer (4.50–3.50 ppm) in methacryloylated derivatives [22,23].

Figure 3.3 shows the infrared spectra for unmodified HEC and HECGMA. The successful modification of HEC with GMA was confirmed by the introduction of a new absorbance band at 1710 cm^{-1} in the HECGMA High spectrum attributed to the stretching frequency of C=O, while absorbance band at 1637 cm^{-}1 is due to C=C groups [25]. In Figure 3.3(b), the band 813 cm^{-1} is the characteristic of CH out-of-plane vibration present in all HECGMA [24,25]. This band results from the presence of methyls of methacrylol groups. It was observed that all the above bands mentioned were present following modification of HEC at a high molar ratio to GMA.



(a) (b)

Figure 3.3. FTIR spectra of unmodified HEC and HEC modified with GMA at a low, medium, and high molar ratio with wavenumbers (a) in the range of 3750–750 cm^{-1}; and (b) at the range of 2000–750 cm^{-1}.

A linear correlation with a regression coefficient R^2 of 0.9993 and a linear equation of y = 114.17x + 4.2548 was obtained for calibration (see Figure 3.4). The retention time (Rt) of the analyzed compound was 25.9 min.

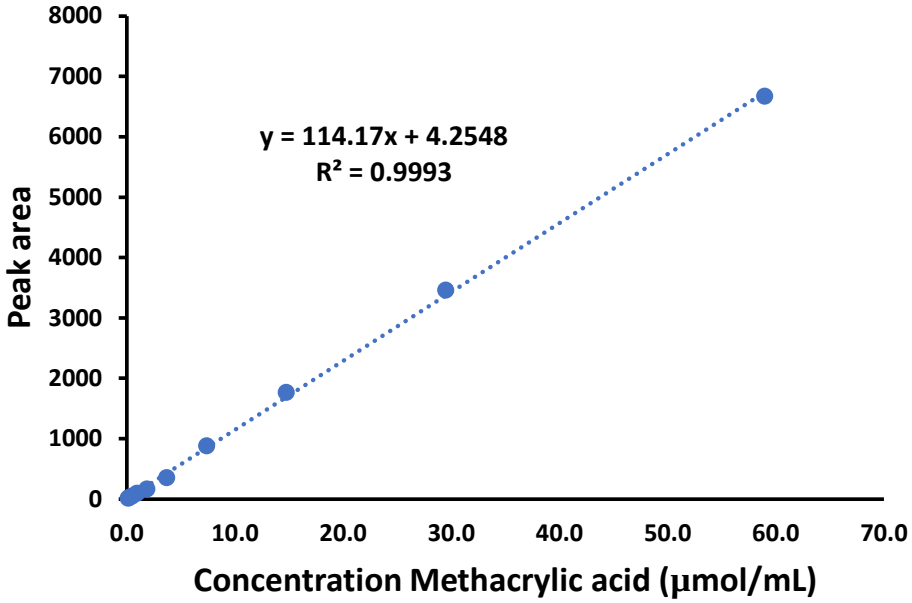


Figure 3.4. Standard calibration curve of methacrylic acid

Quantification of methacrylic acid recovered from hydrolysed modified HEC samples showed that the methacryloyl groups content in HECGMA high, medium and low were 173.50 ± 32.84 µmol/g, 72.43 ± 6.16 µmol/g and 64.49 ± 5.98 µmol/g, respectively (see Table 3.2). The results for negative control (unmodified HEC) show no presence of methacryloyl groups.

Table 3.2. Calculation of amount of methacrylic acid in HECGMA samples

Sample	Peak	Concentration of methacrylic acid (µmol/mL)	Actual amount of methacrylic acid (µmol/g)
HEC	0.00	0.00	0.00
HECGMA low	41.10 ± 0.08	0.32 ± 0.03	64.49 ± 5.98
HECGMA medium	45.60 ± 7.80	0.36 ± 0.03	72.43 ± 6.16
HECGMA high	103.30 ± 23.00	0.87 ± 0.16	173.50 ± 32.84

3.3.2. Acute toxicity assay and fluorescent assay in planaria

Toxicology screening of the HECGMA was performed using fixed-dose procedures on planaria worms. Planaria were used in toxicology screening of chemicals because of their permeable epithelia that may absorb low molecular weight compounds from their environment [29]. The acute toxicity assay using planaria revealed that HECGMA derivatives at the studied concentrations (0.01% w/v, 0.05% w/v, 0.25% w/v, 0.50% w/v and 1.00% w/v) do not cause death in planaria for 24 hours, 48 hours and 72 hours of exposure. The exception is the control group of worms exposed to 1% BAC, which resulted in dead planaria, with no signs of worm movement at all.

Fluorescent assay was previously developed by our research group using planaria model to evaluate the effect of irritant chemicals on the permeability of their epithelial membranes [16]. The assay is based on disruption of planaria epithelia caused by irritant chemicals. When planaria are exposed to an irritant chemical the integrity of their epithelium is disrupted and this facilitates penetration of fluorescein sodium into their body. This is evaluated through the

analysis of fluorescent microphotographs of worms following their exposure first to a chemical of interest, then to solution of sodium fluorescein. Fluorescent assay was carried out to evaluate the effect of 1% (w/v) HEC and HECGMA on planaria epithelia for 24 hours of exposure. Figure 3.5 presents fluorescence images as well as the results of image analysis after 24 hours expressed as fluorescence intensity values. A 24 hours exposure of planaria to different polymers indicated that even unmodified HEC causes a statistically significant enhancement ($p < 0.05$) of fluorescein penetration into the worms' bodies compared to the negative control with artificial pond water (APW). It is well known that HEC is widely used in various topical and mucosal formulations, and it is a biocompatible and non-irritant polymer at this concentration [30]. Exposure of planaria to HECGMA Low and Medium did not cause a significant increase in the fluorescence intensity compared to unmodified HEC ($p > 0.05$); this indicates that these two derivatives have non-irritant properties like HEC. However, exposure of planaria to HECGMA High resulted in a 2× time greater fluorescence intensity compared to unmodified HEC, which indicates that this sample is potentially more irritant.

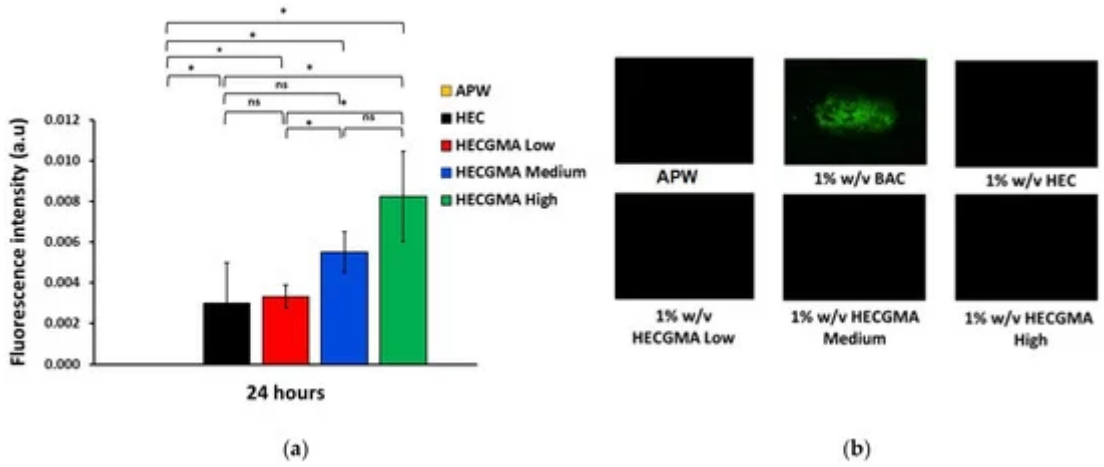


Figure 3.5. Fluorescent assay using planaria. (a) Histograms representing the relative intensity of fluorescence in planaria after the exposure to 1 % (w/v) of unmodified HEC, HECGMA low, HECGMA medium and HECGMA high. (b) Images of planaria worms after exposure to APW, 1% (w/v) of BAC, HEC, HECGMA low, HECGMA medium and 1% HECGMA high. Data show the mean ± SE (n = 3). * Statistically significant according to t-test; $p < 0.05$, ns = not significant.

3.3.3. *In Vitro* cytotoxicity

The cytotoxicity of HEC and HECGMA derivatives was studied using the Caco-2 cell line in a concentration range of 0.05 to 1% (w/v). MTT results showed that the cell viabilities are comparable for HEC and all HECGMA derivatives and all are above 60% after 24 hours (Figure 3.6). In the majority of cases, the difference between the unmodified HEC and HECGMA derivatives was not statistically significant ($p > 0.05$), which indicates that chemical modification of HEC with methacryloyl groups does not cause an increase in polymer toxicity.

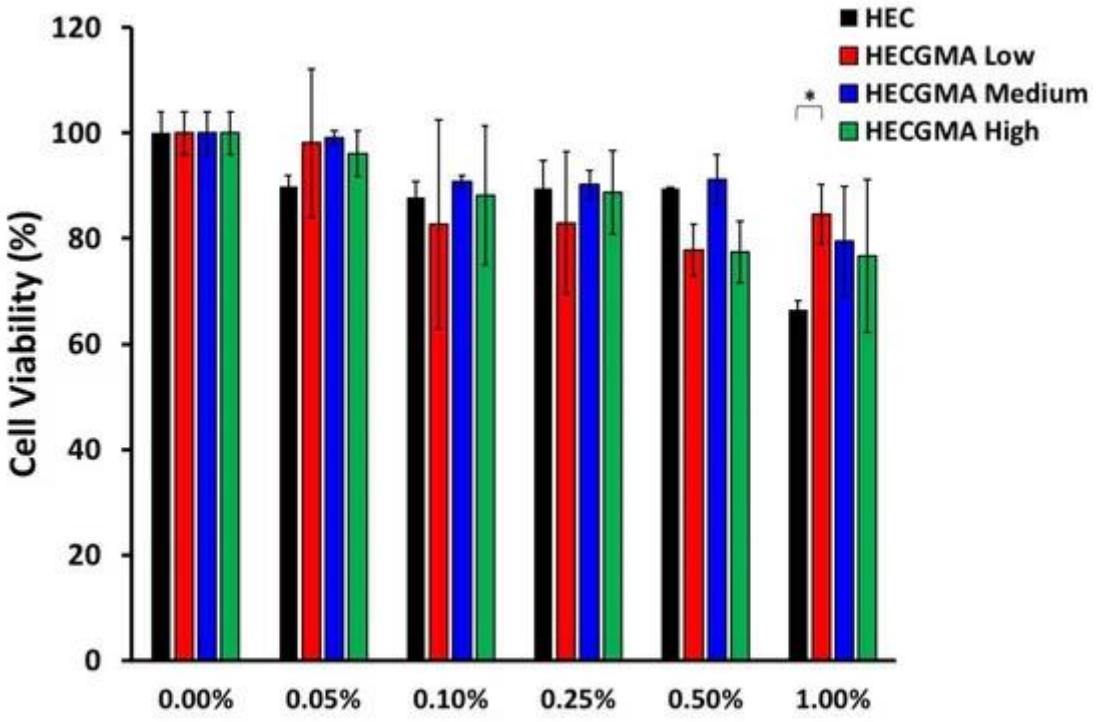


Figure 3.6. Cell viability evaluated using MTT assay with the percentage of viable cells after the exposure to 0%, 0.05%, 0.10%, 0.25%, 0.50%, and 1.00% of HEC and HECGMA derivatives at 24 hours. Data show the mean values ± SD (n = 3). * Statistical significance is shown according to t-test; $p < 0.05$.

3.3.4. Preparation and physical characterisation of wafers

Lyophilized formulations, containing water-soluble polymers, often form wafers that are widely reported in the literature for application in buccal drug delivery. In the present work, the unmodified HEC and new HECGMA derivatives were used to prepare wafers as model dosage forms. The wafers developed in our work were light, spongy and white with a soft and smooth texture. The texture of wafers is important as it influences the oral intake of medicine. Grittiness from the product formulations gives an unpleasant mouthfeel after intake [31]. All

the formulations were easily removed from the mould. Selected images of these wafers are shown in Figure 3.7.

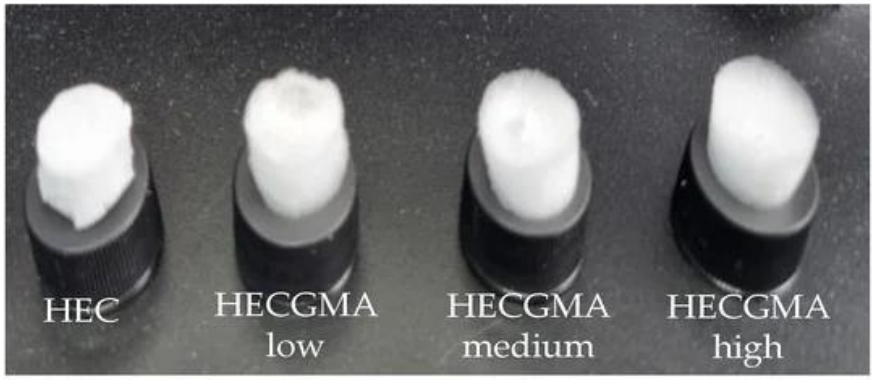


Figure 3.7. Physical appearance of lyophilised wafers based on HEC and HECGMA derivatives.

The average diameter of these wafers was 12 mm. The morphology of the wafers was examined using SEM (Figure 3.8). The porosity of wafers was conferred by freeze-drying as a result of the elimination of ice crystals via the sublimation process [31].

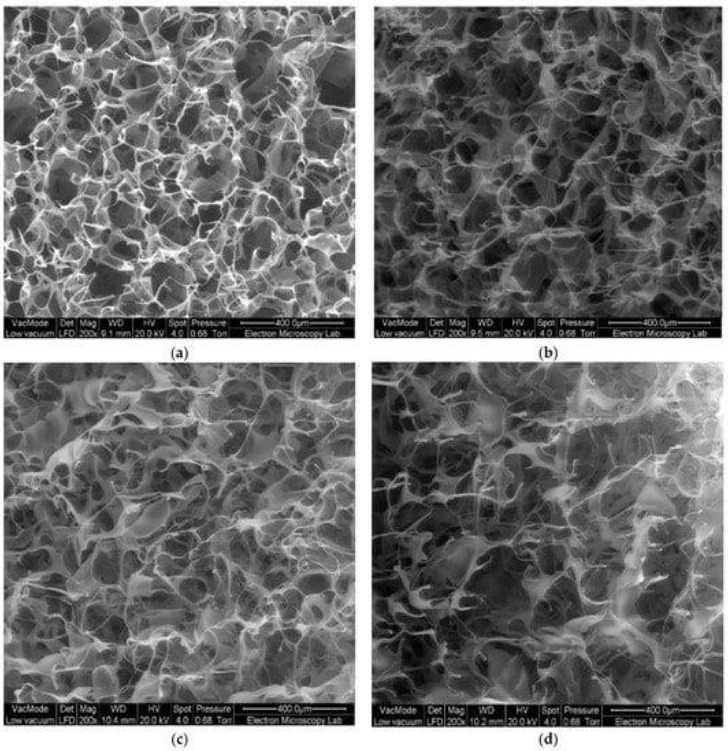


Figure 3.8. SEM images of lyophilised wafers of (a) unmodified HEC, (b) HECGMA low, (c) HECGMA medium and (d) HECGMA high.

3.3.5. *Ex Vivo* evaluation of mucoadhesive properties of wafers

Adhesion of the wafers to freshly excised sheep buccal mucosa was evaluated using a tensile test, established in the literature on mucoadhesive dosage forms [1]. This test provides two main parameters such as the peak force or maximal detachment force and the total work of adhesion, calculated as the area under the detachment curve. Figure 3.9 shows the results of the tensile test evaluating mucoadhesive properties of the wafers, including the data on the peak force and the total work of adhesion. As expected, the wafers prepared from unmodified HEC exhibited relatively modest adhesion because of the non-ionic nature of this polymer [1]. However, a statistically significant improvement in adhesive properties was observed for the wafers prepared from HECGMA derivatives. The adhesive properties generally improve for the derivatives with greater content of methacryloyl groups in the polymer. HECGMA High derivative exhibited the greatest mucoadhesive performance, whose peak force and the total work of adhesion were 3.27× and 3.79× greater compared to these parameters recorded for the wafers composed of unmodified HEC.

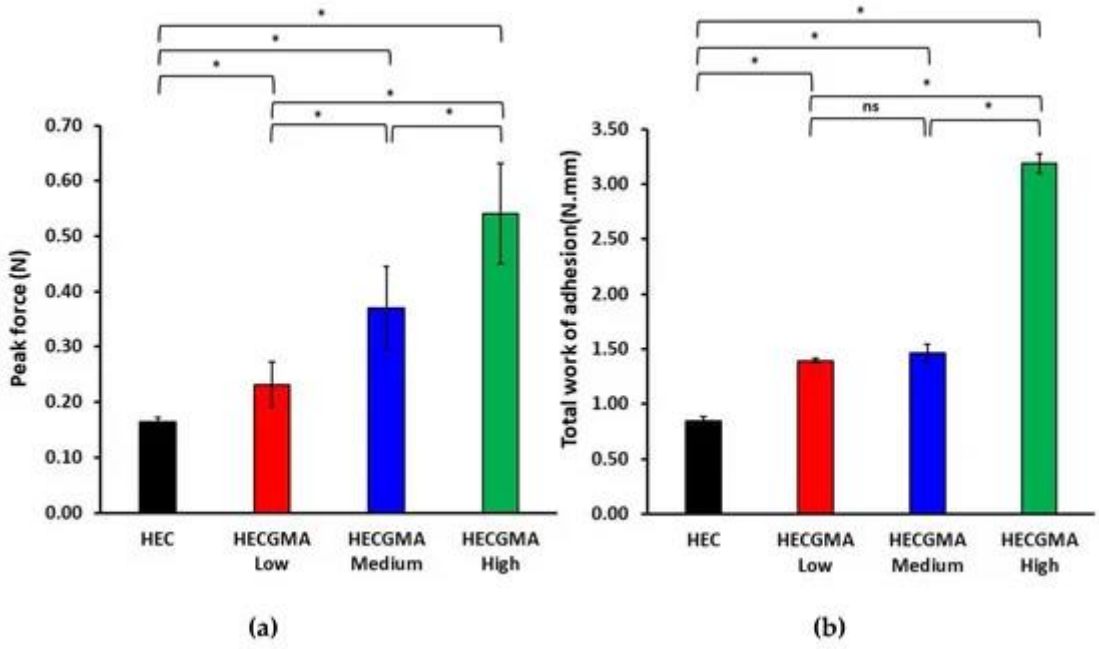


Figure 3.9. The results of tensile test to evaluate mucoadhesive properties of the wafers based on unmodified HEC and HECGMA derivatives: (a) Peak force (N) and (b) Total work of adhesion (mm·N). Data show the mean values ± SD (n = 5). * Statistically significant according to t-test; $p < 0.05$; ns = not significant.

Thus, methacryloylated HEC exhibits enhanced mucoadhesive properties and can be used to formulate dosage forms for buccal drug delivery. The advantage of methacryloylated HEC compared to other mucoadhesive polymers commonly used for buccal delivery such as chitosan [32], sodium carboxymethylcellulose, poly (acrylic acid) derivatives and carragennan [33], pectin [34] and alginates [35] is its non-ionic nature. Non-ionic polymers have better compatibility with ionic drugs as they will not form insoluble complexes that may affect release characteristics.

3.4. Conclusions

The present study demonstrated that poor mucoadhesive properties of HEC could be significantly improved by introduction of methacryloyl groups into the structure of this non-ionic polymer. This was achieved by reaction of HEC with glycidol methacrylate. The structure of resulting HEC derivatives was confirmed using FTIR and ^{1}H NMR spectroscopies as well as by HPLC-based assay to quantify the presence of methacrylic acid in the hydrolysed polymers. The tests performed using planaria and Caco-2 cells indicated that the new HEC derivatives do not show any adverse toxicological reactions similarly to unmodified HEC. All these polymers were then prepared as wafers and their mucoadhesive properties were evaluated using a tensile test in freshly excised sheep buccal mucosal model. All HEC derivatives exhibited superior mucoadhesive properties compared to unmodified HEC and the greater presence of methacryloyl groups improved adhesiveness to mucosa. The new excipients based on HECGMA can be easily synthesised and have solubility in water. Potentially these polymers can be used not only for the preparation of wafers for buccal drug delivery but also for other solid, liquid and semi-solid dosage forms for transmucosal administration.

Glycidol methacrylate is a chemically reactive molecule that can potentially be used for introducing unsaturated functional groups to a variety of hydroxyl-containing water-soluble polymers to enhance their mucoadhesive properties. Modification of these polymers with glycidol methacrylate may offer some advantages compared to the use of methacrylic anhydride as a reagent for derivatisation. Water-soluble polymers modified with glycidol methacrylate may exhibit better hydrophilic properties because of the possibility of reaction via epoxide ring opening.

3.5. References

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Chapter 4.

Hydroxyethyl cellulose functionalised with maleimide groups as a new excipient with enhanced mucoadhesive properties

(QUAD system contribution of Fhataheya Buang: 60% of conception and design, 90% of data collection, 70% of data analysis and conclusions, and 70% of manuscript preparation).

Chapter 4.

Hydroxyethyl cellulose functionalised with maleimide groups as a new excipient with enhanced mucoadhesive properties

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Abstract

Hydroxyethyl cellulose (HEC) is a non-ionic water-soluble polymer with poor mucoadhesive properties. The mucoadhesive properties of hydroxyethyl cellulose can be improved by modifying it through conjugation with molecules containing maleimide groups. Maleimide groups interact with the thiol groups present in cysteine domains in the mucin via Michael addition reaction under physiological conditions to form a strong mucoadhesive bond. This will prolong the residence time of a dosage form containing this modified polymer and drug on mucosal surfaces. In this study HEC was modified by reaction with N-(4-bromophenyl) maleimide in varying molar ratios and the successful synthesis was confirmed using ^{1}H NMR and FTIR spectroscopies. The safety of the newly synthesised polymer derivatives was assessed with *in vivo* planaria assays and *in vitro* MTT assay utilising Caco-2 cell line. The synthesised maleimide-functionalised HEC solutions were sprayed onto blank tablets to develop a model dosage form. The physical properties and mucoadhesive behavior of these tablets were evaluated using a tensile test with sheep buccal mucosa. The maleimide-functionalised HEC exhibited superior mucoadhesive properties compared to unmodified HEC. Graphical abstract as shown in Figure 4.1.

Keywords: hydroxyethyl cellulose; mucoadhesion; maleimide; oral delivery; spray coated tablet

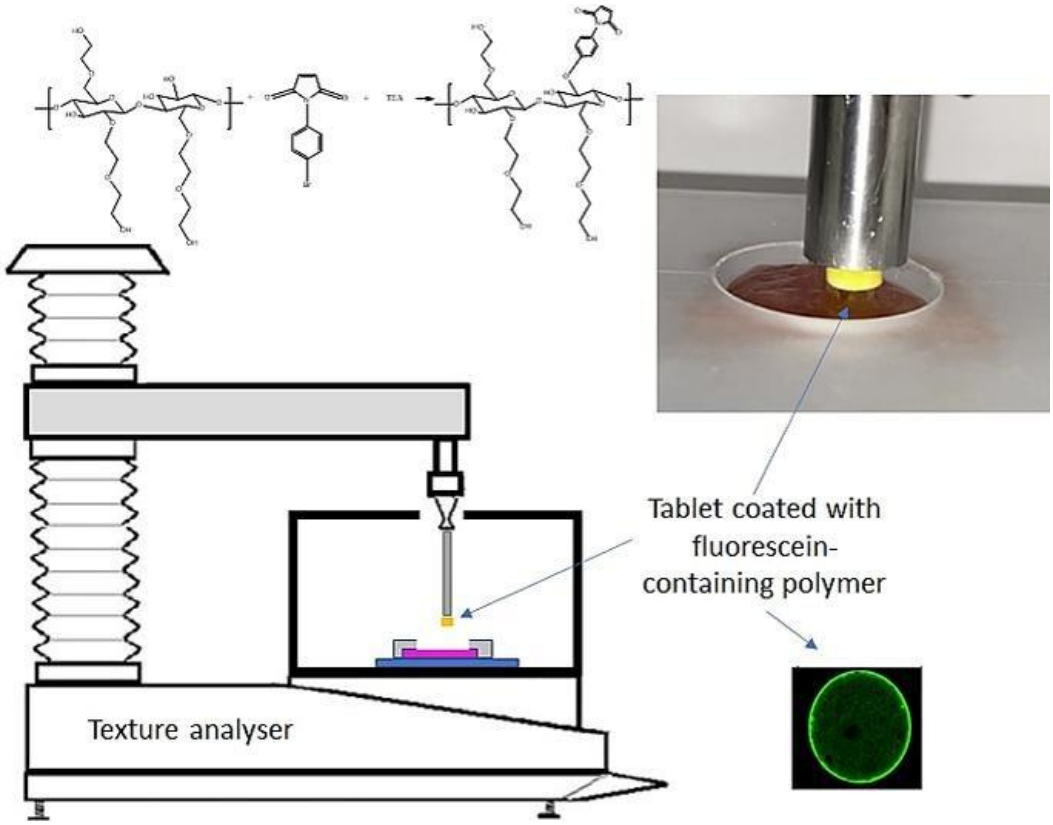


Figure 4.1. Graphical abstract

4.1. Introduction

In modern medicine, excipients play an important role in pharmaceutical drug formulation. US Food and Drug Administration defines excipients as inactive or inert ingredients or substances intentionally added to a drug that is not part of the active substance[1]. Pharmaceutical excipients are typically included in larger quantities in dosage forms and can account for up to 90% of medicinal products [2]. The excipients with added functionalities or 'multifunctional excipients' are subject of recent interest among pharmaceutical manufacturers. The added functionality can be achieved by developing a new excipient by chemical modification, co-processing existing excipients or synthesis of novel materials [3].

Here, we are interested to develop a new excipient from non-ionic hydroxyethyl cellulose (HEC) which is widely used in food and drug formulations as a thickening agent [4]. Enhanced mucoadhesive characteristics is one of the desirable properties to improve in HEC. Mucoadhesion is the ability of materials to adhere to mucosal membranes in the human body and it provides an improved retention on the tissue allowing more efficient absorption of drug molecules [5]. Non-ionic polymers often have poorer mucoadhesive properties compared to polyelectrolyte[5,6].

Enhancement of mucoadhesive properties of non-ionic polymers can be achieved by their chemical modification, which involves the introduction of new functional groups. This enhancement can be accomplished by incorporating functional groups of an ionic nature, thereby transforming a non-ionic polymer into a polyelectrolyte. Alternatively, a non-ionic polymer can be functionalised with groups capable of forming covalent bonds with mucin under physiological conditions. In this case, the mucoadhesive polymer is classified as a mucoadhesive of the second generation. Several approaches are known to make mucoadhesive polymers of the second generation. These include introduction of free thiols [7], phenylboronic acid [8], catechols [9], acryloyls [10], methacryloyls [11], aldehydes [12], maleimides [13] and some other groups [6]. Introduction of thiol groups into water-soluble polymers has become most widely explored strategy to enhance mucoadhesive properties with over 450 papers published and recent translation of this approach into commercial products such as Lacrimera eye drops [6]. However, thiolated polymers have limitations such as stability issues caused by their easy oxidation. Therefore, the development of alternatives to thiolation is of great interest.

Several attempts to improve mucoadhesive properties of HEC have been reported previously. Bernkop-Schnurch research group reported the synthesis of betaine-modified HEC [14] and S-protected thiolated HEC [15]. More recently, we also reported modification of HEC by reaction with glycidol methacrylate that resulted in improved mucoadhesive properties [11].

Our group previously pioneered the use of materials with maleimide groups in the design of dosage forms with enhanced mucoadhesive properties. These include the design of hydrophilic nanogels [13], liposomes [16] and nanoparticles [17] functionalised with maleimide groups. Other research groups have also picked up this idea and developed maleimide-functionalised alginate [18], chitosan [19,20] and carboxymethylcellulose [21]. Maleimide groups are expected to show better stability to oxidation compared to thiols.

HEC is much less reactive than chitosan or carboxymethylcellulose because it contains OH groups for potential conjugation with functional molecules. Nevertheless, in this study we developed a new method for introducing maleimide groups into this polymer by reacting HEC with N-(4-bromophenyl)maleimide. The advantage of the new synthetic approach reported in this study is the preservation of the non-ionic nature of HEC derivatives, which may provide better compatibility with charged drug molecules. The resulting HEC derivatives were fully characterized using FTIR and ^{1}H NMR spectroscopies and elemental analysis. The toxicological properties of these derivatives were assessed using *in vivo* assays with planaria and MTT assay in Caco-2 cell line. Model tablets were prepared and coated with maleimide-

functionalised HEC for subsequent assessment of their mucoadhesive properties using a tensile test.

4.2 Materials and Methods

4.2.1. Materials

HEC 720,000 Da, chitosan low molecular weight (chitosan$_{LMW}$, 50,000-190,000 Da), chitosan medium molecular weight (chitosan$_{MMW}$, 190,000-310,000 Da), chitosan high molecular weight (chitosan$_{HMW}$, 310,000-375,000 Da), triethylamine (TEA), N-(4-bromophenyl) maleimide (4-N-BPM), hydrochloric acid, benzalkonium chloride (BAC) and sodium hydroxide were obtained from Sigma Aldrich Co., Ltd., Gillingham, UK. N,N-dimethylformamide (DMF) was provided by SLS Supplies Ltd., Nottingham, UK.

The cell viability assay utilised the following cell culture materials: DMEM High Glucose (Capricorn Scientific GMbH, Germany), foetal calf serum (GE Healthcare Life Sciences, Chicago, IL, USA), penicillin/streptomycin (Nacalai Tesque Inc., Kyoto, Japan), CellTiter 96 Aqueous MTS reagent powder (Promega Corporation, Wisconsin, USA), and phenazine methosulfate (Thermo Fischer Scientific UK Ltd., Leicestershire, UK). The Caco-2 cells were generously donated by Faculty of Pharmacy, UiTM Puncak Alam (Shah Alam, Selangor, Malaysia). The fresh sheep buccal tissues were obtained from PC Turner Abattoir (Farnborough, Hampshire, UK).

4.2.2. Synthesis of HEC derivatives

HEC derivatives (HECMAL) were synthesised in three different molar ratios of [HEC]:[4-N-BPM] = [1]:[1] (HECMAL$_{low}$), [1]:[2] (HECMAL$_{medium}$) and [1]:[3] (HECMAL$_{high}$). Briefly, 4-N-BPM (857 mg for HECMAL$_{low}$, 1715 mg for HECMAL$_{medium}$ and 2572 mg for HECMAL$_{high}$) was dissolved in 50 mL of DMF. Subsequently, TEA (473 μL) was added, and the mixtures were stirred for 30 minutes at 0 °C. Then, 50 mL of 1% (w/v) HEC solution (prepared in deionised water) was added dropwise in these mixtures. The mixtures were then constantly stirred at 25 °C for 24 hours. The resulting solutions were added to excess of cold ethanol and centrifuged at 10000 rpm (10 minutes) for three times. The precipitates from solution were purified by dialysis against deionised water over 72 hours using a cellulose membrane with molecular weight cut off 12-14 kDa (8 changes of water). Finally, the product was recovered after 3-4 days by freeze-drying using Heto PowerDry LL3000 Freeze Dryer (Thermo Fischer Scientific UK Ltd, Loughborough, UK)

4.2.3. ^{1}H Nuclear magnetic resonance (^{1}H NMR) spectroscopy

NMR tubes with a 5 mm internal diameter were used to record the spectra for HEC and HECMAL solutions (20 mg/mL) prepared in D_2O. The ^{1}H NMR spectra were acquired with a Bruker Nanobay 400 MHz two-channel NMR instrument (Bruker UK Ltd., Coventry, UK). The NMR spectra were analysed using MestReNova (Mnova) Version 6.0.2-5475 (Mestrelab Research, S.L., Spain).

4.2.4. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of HEC and HECMAL were recorded using a Spectrum 100 FTIR Spectrophotometer (Perkin–Elmer UK Ltd., Buckinghampshire, UK) from 4000 to 650 cm^{-1}, with a resolution of 4 cm^{-1}, and accumulation of 16 scans. The data obtained were analysed using Spectrum One software (Perkin–Elmer UK Ltd., Buckinghampshire, UK).

4.2.5. Quantification of maleimide

The degree of substitution (DS) was determined for HECMAL polymers, where all peak integrations were normalized to the peak at 3.0 - 5.0 ppm, which corresponds to HEC. The DS was calculated as the ratio between the integral at 6.93 ppm divided by 2 and the sum of the integral at 3.0-5.0 ppm divided by 16 and the integral at 6.93 ppm divided by 2 as shown in equation 1. Values of 2 and 16 used to divide the peak integrations represent the protons on the maleimide and the HEC, respectively.

$$DS = \frac{\text{IH maleimide}/2}{\text{IH HEC}/16+\text{IH maleimide}/2} \qquad (1)$$

Subsequently, the degree of substitution (DS) of the amino moieties was calculated from the elemental composition according to equation 2. The elemental composition of nitrogen was determined by elemental analysis. An amount of 10mg each of HEC and HECMAL polymers were dried in hot air oven overnight. Samples was sent to Medac Ltd, UK for further analysis.

$$DS = \frac{M_{AGU}.\,N\%}{MN.100-M_{SG}.N\%} \qquad (2)$$

M_{AGU} is the molar mass of anhydroglucose repeating unit, N% is the nitrogen content determined by elemental analysis, MN is the molar mass of nitrogen, M_{SG} is the molar mass of the substituent group [22,23].

4.2.6. *In vitro* Toxicity

4.2.6.1 Cells

Caco-2 cells were utilised to assess the cytotoxicity of each polymer. The cells were grown in DMEM high glucose containing 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin. It was kept in an incubator at 37 °C with 5 % CO_2 in the air and 100% relative humidity. Cells were seeded at a density of 1×10^4 cells per well in 96-well plates.

4.2.6.2.Cell viability assay

The cells were subsequently exposed to varying concentrations of the compounds for 24 hours (1.00%, 0.50%, 0.25%, 0.10%, and 0.05% w/v). The untreated cells in the negative control group were found to be 100 % viable. At the end of each treatment, the growth medium was replaced with fresh portion. Each well received 20 μL of a 5 mg/mL MTT solution (in the dark). The cells were incubated for an additional 4 hours at 37 °C in a humidified 5% CO_2 incubator. DMSO was added, thoroughly mixed, and incubated for another 10 minutes. The absorbance was measured at 540 nm using an Infinite 200 PRO microplate reader (Tecan Group Ltd., Switzerland).

4.2.7. *In vivo* Toxicity

4.2.7.1.Acute toxicity assay

Schmidtea mediterranea planaria were kindly provided by Dr Jordi Solana (Oxford Brookes University). The worms were maintained in artificial pond water (APW) at room temperature in the dark. They were fed chicken liver once per week, and APW was replaced on a weekly basis. APW was prepared as a mixture of the following salts: 5 M NaCl, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M $MgCl_2$ and 1 M KCl were dissolved in 50 mL ultra-pure water (UPW).and further diluted in 20L of UPW. The planaria were fed chicken liver once a week and the APW was changed after each feeding.

Planaria (1.0-1.5 cm long) were placed in 24 wells of the plate culture (one in each well) and 2 mL of different concentrations of 1%-0.05% (w/v) of HECAC and HEC solutions were added. The control group consisted of 1% (w/v) BAC; an ingredient found in many mouthwashes. All the test substances were dissolved in APW. The planaria were kept in the plate in the dark at room temperature. The number of live/dead planaria was recorded after 24 hours. Planarians that did not move after gentle shaking were considered dead.

4.2.7.2.Fluorescence intensity test

The experiment was slightly modified from the procedure previously developed by our group [24]. Following a 24-hour treatment with 1% of the test substances (unmodified HEC and modified HECMAL polymers), the planaria were exposed for 1 minute to a 0.1% (w/v) sodium fluorescein solution in APW. The excess fluorescein solution was then removed from the planaria by immersing them in APW for 15 minutes. Each worm was placed on a microscopy glass slide and then immobilised with a few drops of a 2.0 % (w/v) agarose solution. The microscopy glass slide was placed on level surface of ice flakes (-0.5 to -0.8 °C) until the gel hardened. Fluorescence photos of the worms were captured using a Leica MZ10F stereomicroscope (Leica Microsystems, UK) fitted with a DFC3000G digital camera set at 2.0× magnification, 160 ms exposure time, and gamma 0.7.

4.2.8. Preparation of blank tablets

The blank tablets were prepared by mixing 400 g hydroxypropyl cellulose (HPMC), 400 g microcrystalline cellulose (MCC), and 190 g barium sulfate (BS) in a TURBULA® powder blender mixer / 3d shaker mixer (Willy A. Bachofen Maschinenfabrik, Germany) for 10 minutes, following addition of 10 g magnesium stearate (MS) for another 2 minutes. The powder mixtures were dispensed into a hopper above the tablet compression machine Riva Minipress MII (Riva GB Ltd, Aldershot UK). The mixture was compacted with single die set at an automatic mode with speed of production at 40 tablets/min. Average tablet weight, thickness, diameter and hardness were determined for 20 tablets in every batch. Tablet hardness was assessed using a tablet hardness tester (Copley Scientific Limited, Nottingham UK).

4.2.9. Preparation of spray coated tablets

Solutions of 0.1% (w/v) of HEC, $HECMAL_{low}$, $HECMAL_{medium}$ and $HECMAL_{high}$ were prepared by dispersing the polymers in deionised water, while $chitosan_{low}$, $chitosan_{medium}$ and $chitosan_{high}$ in 0.1 M HCl. 2.5 mg/mL of sodium fluoresceine was added to these solutions before being spray-coated onto blank model tablets using Mini Coater Drier (MCD-2) equipment from Caleva (Dorset, UK). The equipment setting for the experiment was consistent with the agitator at 55 %, fan at 9.5 m/sec, temperature at 40 °C, and pump at 4 rpm. The thickness of the tablets coating was evaluated using fluorescence microscopy and subsequent image analysis with Image J software.

4.2.10. *Ex vivo* mucoadhesion

The TA-XT Plus Texture Analyser (Stable Micro Systems Ltd, UK) with a 5 kg load cell was used to study the mucoadhesive properties of all formulations. Sheep buccal tissue was cut into squares and placed in between a cylindrical device and the top cover. The cover had a circular opening of 20 mm in diameter. The mucosal surface of the tissue was exposed through this opening. The tissues were kept at 37°C using a water bath.

Each tablet was attached to the aluminium probe (12 mm in diameter) using a sticky adhesive tape. Then the probe was lowered, and the table was brought in contact with mucosal tissue. The following test parameters were used: pre-speed test 0.5 mm/s; test speed 0.5 mm/s; post-speed test 1 mm/s; applied force 0.5 N; contact time 60 s; trigger type auto; trigger force auto; and return distance 20 mm.

4.2.11. Statistical evaluation

A two tailed Student t-test with 95% confidence interval as the minimal level of significance was employed as the statistical tool to evaluate the data.

4.3. Results and discussion

Maleimide-functionalised HEC was synthesised by reacting HEC with N-(4-bromophenyl) maleimide according to the reaction scheme shown in Figure 4.2. This reaction was catalyzed by addition of TEA as a base and was conducted in an aqueous solution. Three different derivatives were synthesised with different molar ratios of HEC to 4-N-BPM.

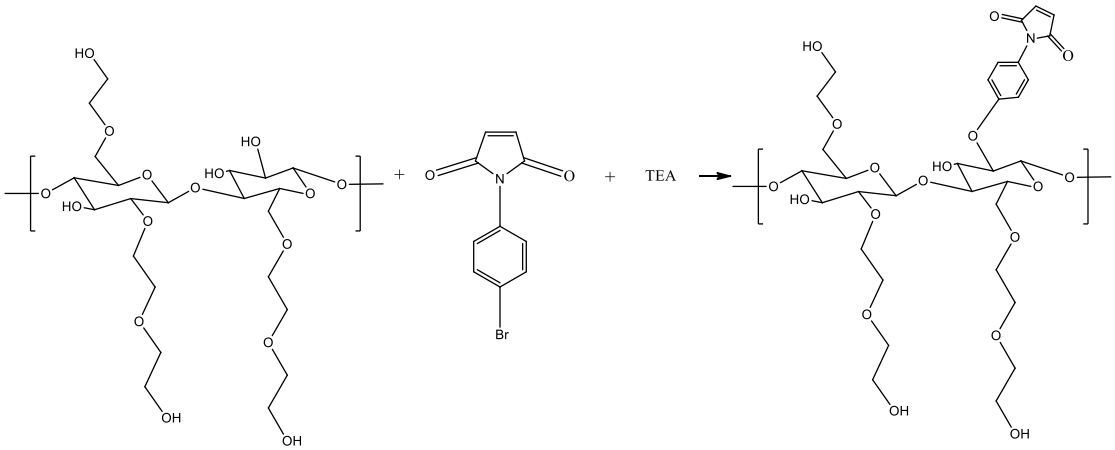


Figure 4.2. Reaction of HEC with N-(4-bromophenyl) maleimide

4.3.1. ^{1}H NMR spectra

The ^{1}H NMR spectra of unmodified HEC and HECMAL samples recorded in D_2O are shown in Figure 4.3. The spectral data confirmed that the synthesis of HECMAL was successful with the presence of signals from the protons that belong to maleimide moieties at 6.93 ppm. The spectrum of 4-N-BPM recorded in DMSO-d6 can be found in Appendix IV. The signals of the aromatic group are detected in the spectra at 7.65 -7.63 ppm, 7.51 -7.49 ppm, 7.35-7.33 ppm and 7.20 – 7.18 ppm. We also found additional signals at 6.50-6.30 ppm. According to Morrison et al. (2019) there is likely an opening of some of maleimide rings in the resulting product with the presence of water that contributes to additional signals in the spectra [25]. This is in agreement with Barradas et al. (1976), who reported that maleimide ing may undergo hydrolysis in alkaline media [26]. Thus, in this reaction, there could be a product with intact and ring-opened maleimide groups as shown in Figure 4.3. The other remaining signals are attributed to the backbone HEC including the peaks at 1.21 ppm and 1.83 ppm with unidentified structure similarly found and reported in D'Avino et al.(2022) and Ray et al., 2018 [27,28].

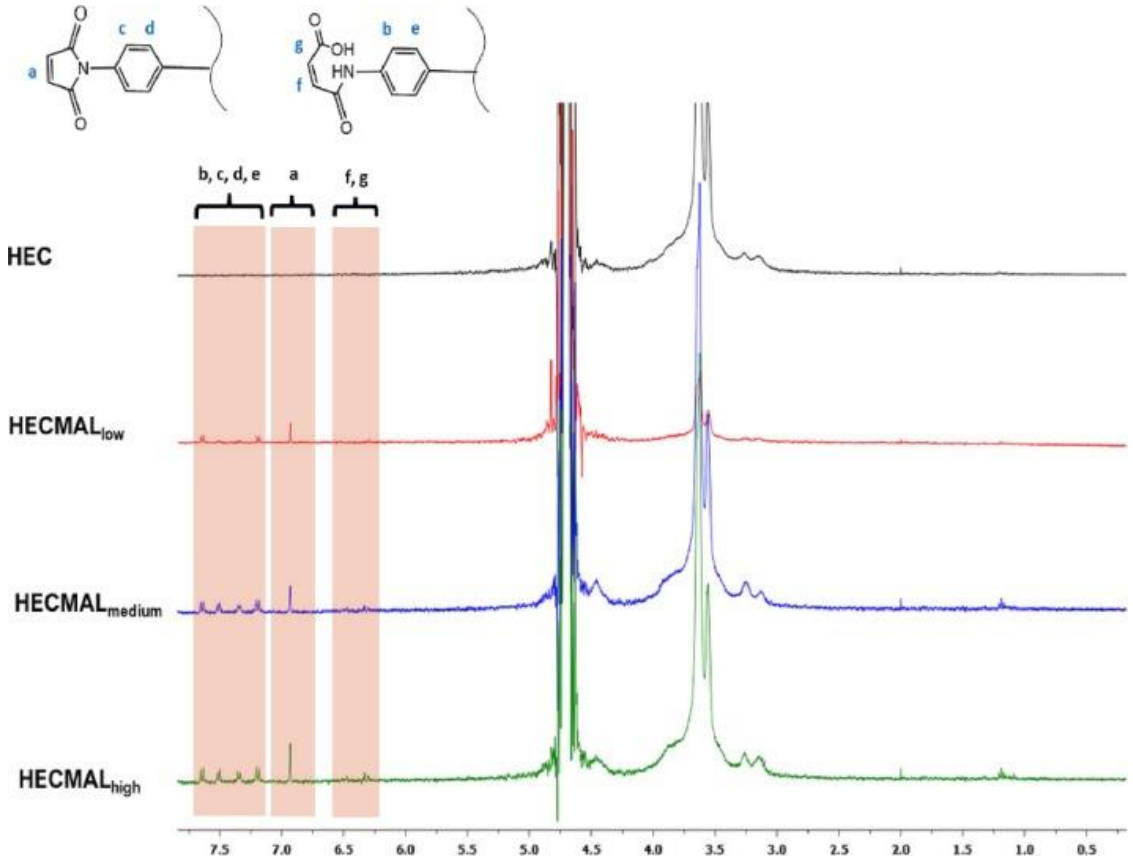


Figure 4.3. ^{1}H NMR spectra of unmodified HEC and HECMAL derivatives recorded in D_2O

4.3.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used for further evaluation of the chemical structures before and after the functionalisation of HEC. Figure 4.4 shows the FTIR spectra of both unmodified HEC and HECMAL derivatives. There are two new bands appear at around 1506 cm^{-1} and 1377 cm^{-1} in the spectra of HECMAL derivatives. These bands confirm the successful introduction of maleimide group into HEC as they represent benzene ring (1506 cm^{-1}) and C-N stretching of maleimide groups (1377 cm^{-1}). Additionally, C=O band appeared at 1703 cm^{-1} is attributed to maleimide ring. The intensity of these bands increased in the spectra with increase in [4-N-BPM]/[HEC] molar ratio.

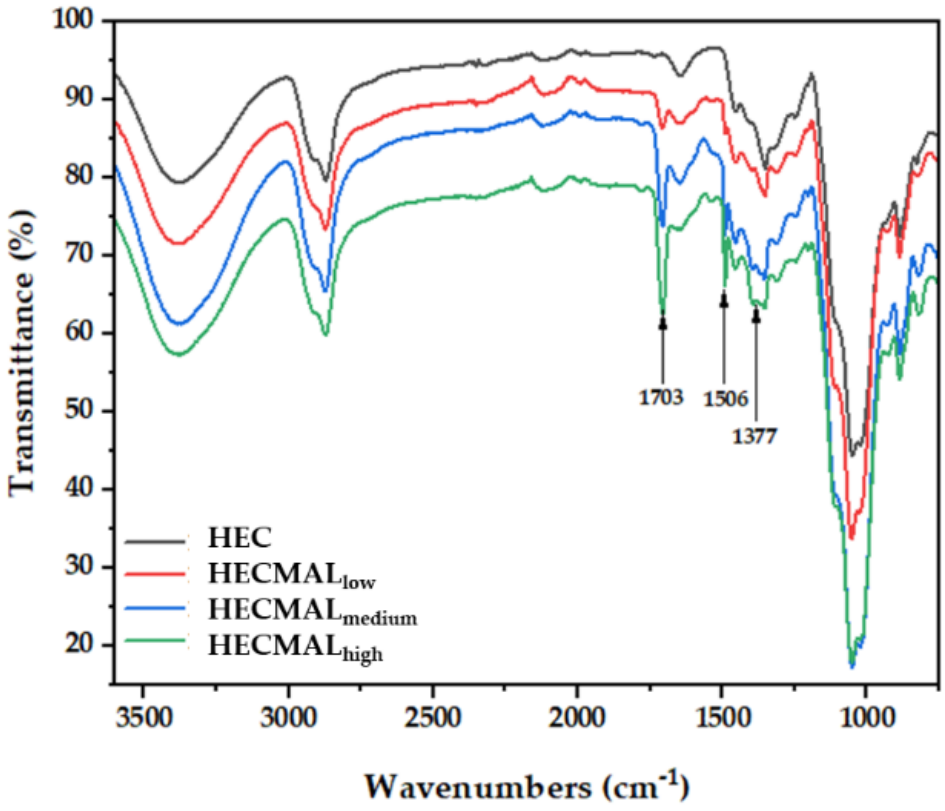


Figure 4.4. FTIR spectra of unmodified HEC, $HECMAL_{low}$, $HECMAL_{medium}$ and $HECMAL_{high}$

4.3.3. Quantification of maleimide groups in HECMAL

The values of DS for HECMAL derivatives determined using two independent methods (NMR and elemental analysis) are summarized in Table 4.1. The DS values are in good agreement with the compositions of the reaction mixtures and as expected show an increase from $HECMAL_{low}$ to $HECMAL_{medium}$ to $HECMAL_{high}$. The results from elemental analysis revealed that the nitrogen content of the modified HECMAL polymers increased as the molar ratio of

4-N-BPM to HEC increased. The higher the molar ratio, the greater the nitrogen content in the polymer, which is related to the presence of maleimide groups. The nitrogen content ranges from 0.43% to 1.35%, with the estimated DS increases from 0.07 to 0.22. Comparable data on DS values were also calculated from ^{1}H NMR with the estimate DS increases from 0.08 to 0.23. The calculation can be found in Appendix III, Appendix IV, Appendix V and Appendix VI.

Table 4.1. Percentage of nitrogen content and DS of HEC and HECMAL

	N%	$^{1}DS_{EA}$	$^{2}DS_{NMR}$
HEC	0.00	0.00	0.00
$HECMAL_{low}$	0.40 ± 0.05	0.07± 0.01	0.08
$HECMAL_{medium}$	0.62 ± 0.01	0.11± 0.00	0.13
$HECMAL_{high}$	1.22 ± 0.19	0.22± 0.03	0.23

$^{1}DS_{EA}$ calculated from elemental analysis; $^{2}DS_{NMR}$ calculated from ^{1}H NMR

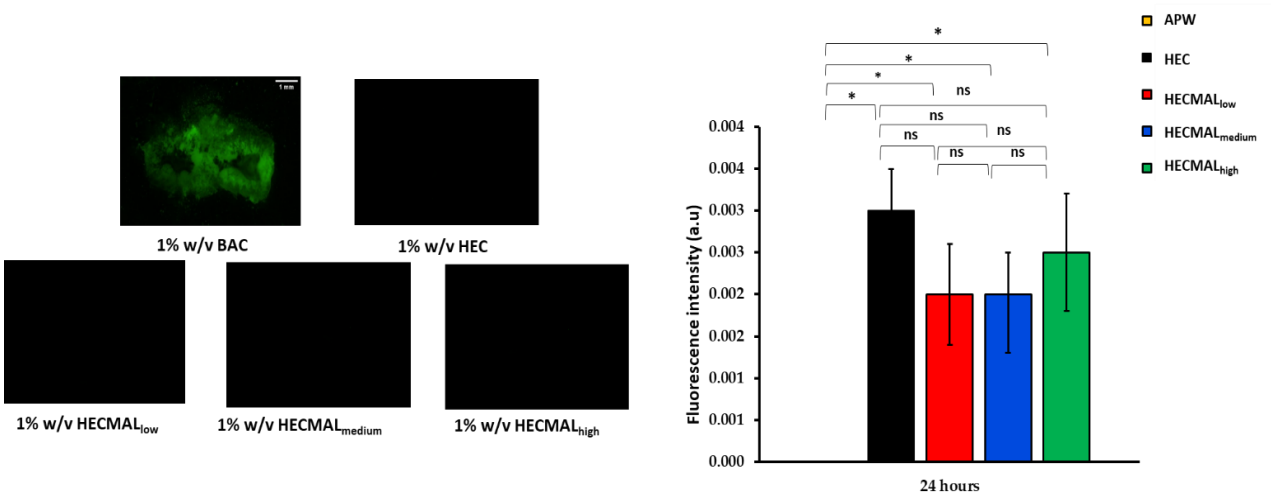
4.3.4. Toxicity evaluation in planaria

HEC has a well-established safety profile and is commonly used as a polymeric excipient for mucosal applications, for example, in ocular [29] and vaginal drug delivery [30]. However, when a pharmaceutically acceptable polymer is modified chemically, its toxicological properties should be extensively evaluated before it can be introduced as a new pharmaceutical excipient. An initial toxicological evaluation of new chemicals can be conducted using invertebrate models such as drosophila, brine shrimp, slugs [31–34]. Previously, we also proposed to use planaria as a rapid and cheap pre-screening tool for potential skin irritants [24] and more recently this model was used to evaluate HEC and methacryloylated HEC for mucosal delivery [11]. In this study, we evaluated the toxicological properties of HEC and HECMAL using planaria *in vivo* assays.

Acute toxicity of live-dead assay was conducted with planaria 24 hours exposure to 1% v/w polymer solutions. No mortality was observed in all tested polymers. All planarian worms were alive, and a few were seen adhering to the side of the walls of the well plates. Ireland et al reported that *S. mediterranea* species have a preference to attach to the side of the wall and have less motility [35].

An additional evaluation of the polymer effects on planaria was conducted using fluorescent assay, which determines the effect of chemicals on planarian body wall integrity and barrier function. When planaria are exposed to irritant chemicals this causes a damage in

their epithelia and reduces their barrier function with respect to small molecules. When planaria are subsequently exposed to a solution of sodium fluorescent, this dye penetrates their body and the extent of penetration can then be assessed using fluorescence microscopy to provide quantitative information [24]. The results of the fluorescent assay following the worms' exposure to HEC and HECMAL solutions are presented in Figure 4.5. An exposure of planaria to HECMAL solutions for 24 hours did not reveal any statistically significant reduction in the epithelial barrier function compared to HEC ($P > 0.05$). This result indicates that the polymers bearing maleimide functional groups do not exhibit any increase in their irritation properties compared to parent HEC. However, when planaria were exposed to strongly irritant 1% (w/v) BAC solutions as a positive control for just 1 hour, statistically significant increase in the fluorescence intensity was observed due to enhanced penetration of sodium fluorescein into their body.



(a) (b)

Figure 4.5. Fluorescence intensity test using planaria: (a) exemplar fluorescent images and (b) fluorescence intensity values calculated using analysis of fluorescent images, following 24 hours exposure of planaria to 1% (w/v) solutions of HEC, HECMAL$_{low}$, HECMAL$_{medium}$, HECMAL$_{high}$ and 1 hour exposure to 1 % (w/v) BAC, with subsequent immersion of the worms in 0.1% (w/v) sodium fluorescein. Each experiment was performed using 3 different worms (n=3) and mean fluorescence intensity values ± standard deviations were calculated. *Statistical significance was determined using t-test and significant differences are shown with * when $p < 0.05$

4.3.5. *In vitro* MTT toxicity test

The MTT test was performed to assess the levels of viable cells following their 24 hours exposure to solutions of HEC and HECMAL at various concentrations (0.05, 0.1, 0.25, 0.5 and 1 % w/v). Caco-2 cell line was chosen for these experiments because this originates from a mucosal tissue (human colon) and is often used for toxicological assessment of pharmaceutical materials for transmucosal applications [36,37]. Figure 4.6 presents the data on cell viability in the presence of HEC and HECMAL. Exposure of Caco-2 cells to all polymer samples tested did not result in a dramatic reduction in their viability in the studied concentration range (0 – 1 % w/v); the levels of cell viability remained high (>66.57 %) even at the highest polymer concentration (1 % w/v). As expected, an increase in the concentration of polymers in solutions resulted in some reduction in the cell viability; however, the viability data generated for 1 % (w/v) HEC in the present work (66.57±5.14) are somehow lower than the results reported by Leonaviciute et al. (2016) (~96 %) [15]. This deviation from the literature data may be related to the difference in the molecular weights of HEC used in our study (HEC MW 720,000 Da) and Leonaviciute et al. (2016) study (HEC MW 250,000 Da) [15]. There was no statistically significant difference between the cell viabilities observed for all polymer samples in the concentration range of 0.05 – 0.5 %. The results generated for HECMAL samples indicate that cell viabilities are above 80% when compared to the unmodified control cells. These results are also in agreement with the study of Pornpitchanarong et al, 2022, who reported non-toxic nature of carboxymethylcellulose functionalised with maleimide groups with using HGF-1 cells [21]. The results of the study of HECMAL in cell culture are in good agreement with the findings from the experiments with planaria. It can be concluded that the newly synthesised HECMAL is non-toxic and suitable for further development in pharmaceutical applications.

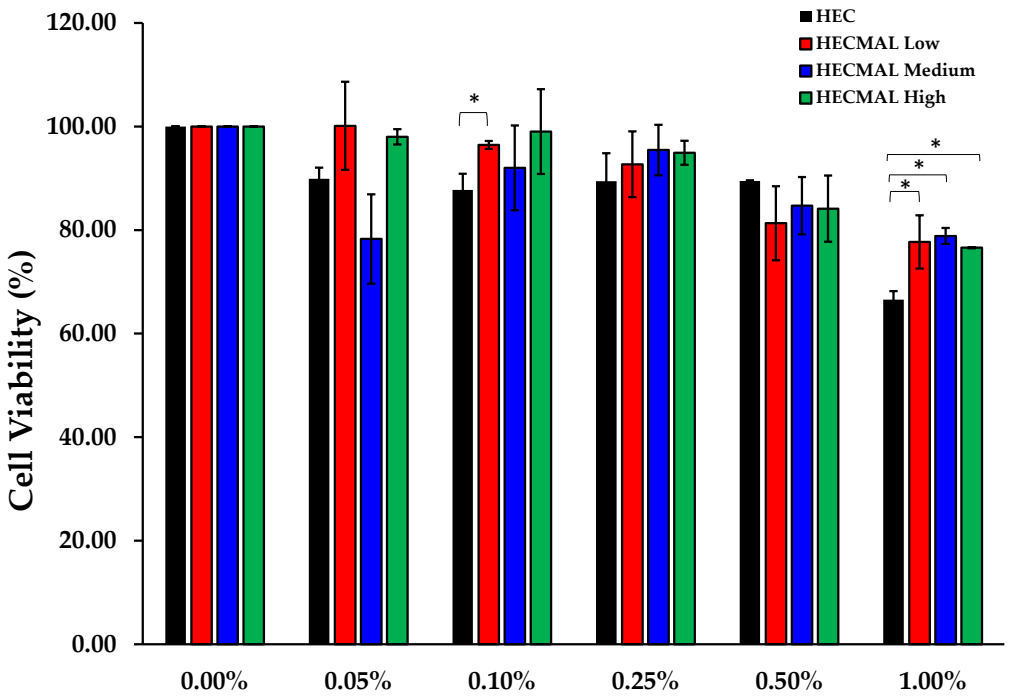


Figure 4.6. Caco-2 cell viability evaluated using MTT assay following their exposure to HEC and HECMAL samples for 24 hours. Data shows the mean ± standard deviations (n = 3). *Statistical significance was determined using t-test and $p < 0.05$ was statistically significant

4.3.6. Design of model tablets coated with different polymers

Blank round tablet formulations were designed as a model dosage form for their subsequent coating with HEC and HECMAL. It is more reasonable to coat tablets with polymers that possess improved mucoadhesive properties, rather than manufacturing tablets using direct compression of these materials. The mucoadhesive polymer present within the tablet bulk does not significantly contribute to the enhancement of mucoadhesive properties, as it primarily acts on the tablet's surface.

The blank tablets were prepared by mixing hydroxypropyl cellulose, microcrystalline cellulose, barium sulfate as bulking agents and binders, with addition of magnesium stearate as a lubricating agent and subsequent compression of powder mixtures. This specific composition was chosen to prepare tablets that will not exhibit any quick disintegration or swelling in an aqueous medium. These tablets were subsequently spray coated with 0.1 % (w/v) HEC or HECMAL or chitosans of different weights mixed with sodium fluorescein using spray coating. Sodium fluorescein was used in this case to facilitate evaluation of the efficiency of their surface coating with mucoadhesive polymers. Chitosans of different molecular weights

(low, medium and high) were used as a positive control due to well documented excellent mucoadhesive properties of this polysaccharide [5,6].

The average weight of these blank model tablets was 102 ± 1 mg and their diameter was 6.00 ± 0.05 mm and their thickness was 3.00 ± 0.04 mm. The hardness of the tablets was found to be 140 ± 1 N. Figure 4.7 shows the fluorescent microscopy images of these model tablets. Image analysis helped to establish that in all cases the tablets were fully and homogeneously coated. However, slightly different polymer thicknesses were observed despite the use of the same spray coating amount and time. Tablets coated with HEC displayed the thickest coating (0.33 ± 0.01 mm), followed by HECMAL at 0.15 ± 0.01 mm and chitosan at 0.11 ± 0.01 mm.

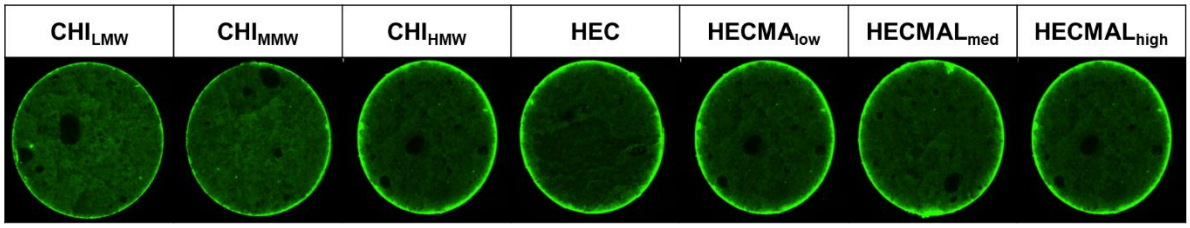


Figure 4.7. Fluorescent images of tablets spray coated with various polymer solutions containing 0.1% sodium fluorescein.

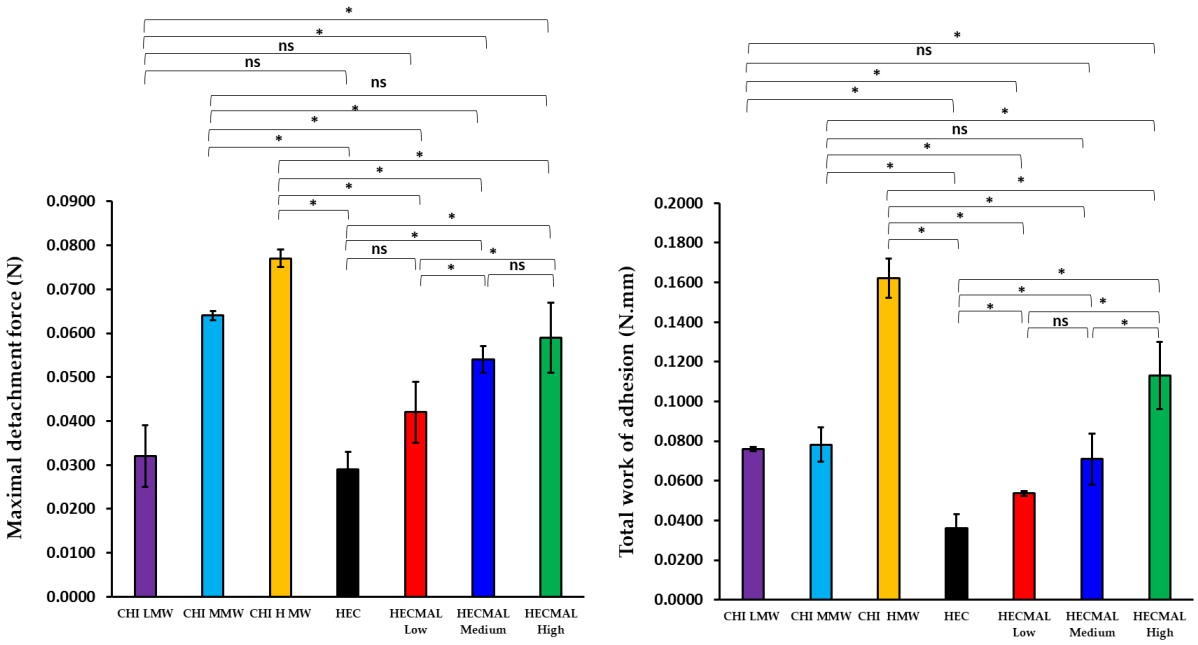
4.3.7. Mucoadhesive properties of tablets coated with different polymers

Mucoadhesive properties of the tablets coated with different polymers were studied with respect to freshly excised sheep buccal mucosa using a tensile test *ex vivo* [5]. This test involves the placement of each tablet in contact with mucosal tissue with its subsequent withdrawal and recording of detachment profiles. Usually, this experiment provides two important characteristics of mucoadhesion through the analysis of each detachment profile: the maximal force observed on the detachment profile is called maximal force of detachment, and the area under the curve provides the total work of adhesion.

Figure 4.8 shows both the values of maximal detachment force (F_{det}) and total work of adhesion (W_{adh}) calculated from these tensile test experiments. The tablets coated with high molecular weight chitosan exhibited superior mucoadhesive properties compared to all other tablets (F_{det} = 0.077±0.002 and W_{adh} = 0.162±0.010). This was expected as chitosan has well documented excellent mucoadhesive characteristics [5] and increase in the polymer molecular weight typically results in improvements in its mucoadhesive performance [38]. Indeed, tablets coated with medium and low molecular weight chitosan exhibited substantially lower F_{det} and W_{adh} values. This decrease in mucoadhesive performance could be related to poorer ability of shorted macromolecules to form entanglements with biomacromolecules of mucins.

Tablets coated with unmodified HEC exhibited the poorest mucoadhesive properties (F_{det} = 0.024±0.004 and W_{adh} = 0.036±0.007). This was also expected because HEC is a non-ionic polymer [5]. An introduction of maleimide groups into HEC structure improves the mucoadhesive properties of the tablets coated with HECMAL dramatically and this property correlates very well with the degree of polymer substitution (DS). Tablets coated with HECMAL samples with greater DS exhibit superior mucoadhesive properties. This is related to the ability of maleimide groups to form covalent linkages with thiol groups present in the mucins via thiol-ene click Michael addition reactions happening under physiologically relevant conditions [39,40].

Although there is a substantial improvement in mucoadhesive properties of tablets coated with HECMAL polymers, the values of F_{det} and W_{adh} for all HECMAL polymers are still lower than similar characteristics recorded for the tablets coated with high molecular weight chitosan. Perhaps the cationic nature of chitosan as well as its high molecular weight for $chitosan_{HMW}$ provides it superior performance [5].



(a) **(b)**

Figure 4.8**.** Mucoadhesive characteristics of tablets coated with different polymers: (a) Maximal detachment force and (b) Total work of adhesion.
Data show the mean values ± standard deviations (n = 3). *Statistically significant difference when $p < 0.05$

4.4 Conclusions

This study reveals that modification of HEC with maleimide moieties results in formation of polymers with improved mucoadhesive properties. The properties of these polymers can be tailored by varying the molar ratio of N-(4-bromophenyl)maleimide to HEC in the reaction mixture. HEC with greater concentration of maleimide groups exhibits superior mucoadhesive properties. The introduction of maleimide groups into these polymers is not detrimental to their toxicological characteristics as evaluated using *in vivo* planaria model and *in vitro* cell culture assay in Caco-2 cells. HECMAL polymers can be considered as a new excipient for formulation of mucoadhesive dosage forms for transmucosal drug delivery. In this work, we have demonstrated the use of these polymers to enhance the mucoadhesive properties of tablets through their surface coating. However, HECMAL can also find applications in other areas of transmucosal drug delivery, for example, when formulated as gels, films and nanoparticles.

4.5. References

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Chapter 5.
Synthesis of acryloylated hydroxyethyl cellulose (HEC) as a new polymer with enhanced mucoadhesive properties

(QUAD system contribution of Fhataheya Buang: 60% of conception and design, 70% of data collection, 70% of data analysis and conclusions, and 70% of manuscript preparation).

Chapter 5

Synthesis of acryloylated hydroxyethyl cellulose (HEC) as a new polymer with enhanced mucoadhesive properties

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Abstract

Mucin glycoproteins are the part of mucus that lubricates and protects the oral cavity. The thiol group (S-H) in mucin can be covalently bonded to an acrylate end group in a physiological environment via the Michael addition reaction. This interaction creates a strong bond between the polymer and the mucosal surface. Thus, this study aims to determine the mucoadhesive properties of modified non-ionic HEC with acryloyl chloride containing an acryloyl group. The successful modification was characterised by ^{1}H NMR and FTIR spectroscopy and the amount of acryloyl groups was quantified by HPLC and elemental analysis. The newly modified HECAC polymers were confirmed to be non-toxic and safe by *in vitro* cell viability tests with Caco-2 cells and *in vivo* toxicity tests using planaria worms. The results from tensile tests showed that all modified HECAC polymers had improved mucoadhesive properties compared to native HEC. The modified HECAC polymers and mucin mixtures have shown a positive synergistic effect in rheology studies.

Keywords: hydroxyethyl cellulose; mucoadhesion; acryloyl; michael addition; films

5. Introduction

Drug administration through the mucous membranes of the body is a non-invasive method of achieving both a local and systemic effect. This route of administration has advantages such as a longer release time of the drug, higher bioavailability and avoidance of the first-pass effect or pre-systemic metabolism [1–3].

The mucosa lining the structures within the oral cavity is called the oral mucosa. Drug delivery through the mucosa is challenging because between 0.5 and 1.5 l of saliva per day is constantly produced by the salivary glands [4]. The mucus in saliva contains an important component called mucin, which forms an important protective layer on the moist epithelial surfaces of the body. It is reported that saliva has a mucin concentration of about 200 µg/ml [5]. For buccal delivery, film has several advantages as a dosage form e.g. it is more pliable and adapts better to the mucosal surface, stays longer at the site of application and has better patient compliance [6].

In 2010, Bianco-Peled et al. extended the idea of Hubbell et al. that any polymer bearing an electronegative unsaturated group can bind covalently to electronegative neighbouring groups, such as sulfhydryl-containing biomolecules like mucin glycoprotein via Michael addition and form a strong mucoadhesive bond [6]. Unsaturated groups such as acrylates and maleimides are known to be reactive groups for Michael addition [7]. Bianco Peles's group had successfully synthesised acrylated chitosan and Pluronic® F12 and showed both synthesised polymers improved the mucoadhesive properties of unmodified polymers [8,9]. Following these publications, our group synthesised acryloylated polymers with Eudragit and PDMAEMA and supported the work [10,11]. Recently, we have published some papers on the modification of non-ionic HEC with methacryloyl and maleimide groups and significantly improved the mucoadhesive properties of non-ionic HEC [12,13].

HEC is a widely used excipient that acts as a binder and thickener in pharmaceutical dosage form. It is a non-toxic, water-soluble polymer with poor mucoadhesive properties. Thus, in this study, we aimed to functionalise non-ionic HEC with acryloyl groups to improve the mucoadhesive properties. The HEC derivatives (HECAC) were characterised by ^{1}H NMR and FTIR spectroscopy and quantified with HPLC and elemental analysis. A simple rheology study was performed to evaluate the interaction between mucin and polymers. The safety of the HECAC polymers were investigated using *Schmidtea mediterranea* planarian worms and in the MTT toxicity assay on Caco-2 cells. The blank film was prepared using HECAC and its mucoadhesive profiles were evaluated with freshly excised sheep nasal mucosa using a texture analyser.

5.2. Material and Methods

5.2.2. Materials

HEC 720,000 kDa, triethylamine (TEA), Trifluoroacetic acid (TFA), acryloyl chloride (AC), hydrochloric acid (HCl), sulfuric acid, acrylic acid, and benzalkonium chloride were purchased from Sigma Aldrich Co Limited, Gillinghamm UK. N, N-Dimethylformamide (DMF) was supplied by SLS Supplies Limited, Nottingham, UK. All reagents used in the study were of analytical reagent grade.

Cell culture materials DMEM High Glucose (Capricorn Scientific GMbH, Germany), fetal calf serum (GE Healthcare Life Sciences, Chicago, IL, USA), penicillin/streptomycin (Nacalai Tesque Inc., Japan), CellTiter 96 Aqueous MTS reagent powder (Promega Corporation, Wisconsin, USA) and phenazine methosulfate (Thermo Fisher Scientific, Massachusetts, USA), were used for cell viability assay. The Caco-2 cells were donated by Dr Sharifah Aminah from the Faculty of Pharmacy, UiTM Puncak Alam, Malaysia.

The excised upper and lower lips of the sheep were obtained from a local abattoir PC Turner Abattoir (Farnborough, Hampshire, UK).

5.2.2. Synthesis of acryloylated HEC

1% (w/v) solution was prepared by dissolving HEC in trifluoroacetic acid. After the mixture was completely mixed, TEA was added as a catalyst. AC was added at different molar ratios (low, medium and high) and stirred constantly at 25°C for 24 hours. The low molar ratio is [HEC]:[AC] = 1:1, the medium molar ratio is [HEC]:[AC] = 1:3 ratio and the high molar ratio is [HEC]:[AC] = 1:6. The reaction product was purified by dialysis against deionised water using dialysis membrane (12-14 kDa). The DIW was changed 8 times (4.5 L) per day for 48 hours. The final product was obtained by freeze-drying.

5.2.3. ^{1}H Nuclear magnetic resonance (^{1}H NMR) spectroscopy

The solutions of HEC and HECAC were prepared in D_2O solvent in 5 mm diameter NMR tubes. The ^{1}H NMR spectra were performed on a 400 MHz Ultrashield Plus™ B-ACS 60 spectrometer (Bruker UK Ltd., Coventry, UK.). The spectra were analysed using MestReNova (Mnova) Software Version 6.0.2-5475 (Mestrelab Research, S.L., Spain).

5.2.4. Fourier transform infrared (FTIR) spectroscopy

The spectroscopic analysis of the HEC and HECAC were scanned from 4000 to 650 cm^{-1}, resolution of 4 cm^{-1} and accumulation of 16 scans using Spectrum 100 FT-IR Spectrophotometer (Perkin–Elmer UK Ltd., Buckinghamshire, UK.). The collected data were generated from Spectrum One software (Perkin–Elmer UK Ltd., Buckinghamshire, UK.).

5.2.5. High-performance liquid chromatography (HPLC)

Acrylic acid was extracted from the film samples by hydrolysis of 40 mg of HECAC polymer with 0.01M sulfuric acid and refluxed at 50°C for 4 hours . The HPLC procedure was adopted from Paleologos and Kontaminas, 2005 and was performed on an Agilent Infinity 1200 HPLC system with an Aminex 87H (Biorad, Watford, UK) column at 40°C [14]. Isocratic elution was performed at 0.6 mL min^{-1} with 0.01M sulfuric acid solution. The acrylic acid was detected using a diode array detector (DAD) (Agilent Infinity 1200 Series, Didcot, UK) at 200 nm wavelength.

The standards (acrylic acid) were dissolved in 0.01M sulfuric acid to make the standard stock solutions and diluted to the final concentrations (concentrations ranging from 0.1 to 72.9 µmol/mL) to generate external calibration curves for further quantification of acrylic acid in the samples (see Appendix VII).

5.2.6. *In vitro* Toxicity

5.2.6.1. Cells

Caco-2 cells were used to determine the cytotoxicity of all the polymers. The cells were cultured in DMEM High Glucose supplemented with 10 % fetal calf serum and 1 % penicillin/streptomycin. It was maintained in a 37^0C incubator with 5 % CO_2 in the air and 100% relative humidity.

5.2.6.2. Cell viability assay

Caco-2 cells were used to evaluate the cytotoxicity of all polymers. Caco2 cells were cultured in DMEM High Glucose supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. They were maintained in a 37°C incubator with 5% CO_2 and 100% relative humidity. Cells were seeded at a density of 1 x 10^4 cells per well in 96-well plates and incubated overnight at 37°C in humidified air containing 5% CO_2.

The cells were then treated for 24 hours with various concentrations of the test substances (1.00% 0.5 0%, 0.25 %, 0.10 %, and 0.05 % w/v). The negative control group consisted of

untreated cells that were considered 100% viable. The media were replaced with a new growth medium after each treatment. Each well received 20 uL of a 5 mg/mL MTT solution (in the dark). The cells were incubated for further 4 hours at 37°C in a humidified 5% CO_2 incubator. DMSO was then added, mixed thoroughly and incubated for a further 10 minutes. Absorbance (Abs) was measured at 540 nm using an Infinite 200 PRO microplate reader (Tecan Group Ltd., Maennedorf, Switzerland).

5.2.7.*In vivo* Toxicity

5.2.7.1. Planarian acute toxicity assay

Schmidtea mediterranea planarian worms were donated by Oxford Brookes University and were kept and maintained in artificial pond water (APW) at room temperature. APW was prepared with different salt concentrations (5 M NaCl, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M $MgCl_2$ and 1 M KCl diluted in 50 mL ultra-pure water (UPW) and further dissolved in 20 L of UPW). The planaria were fed chicken liver once a week and the APW was changed after each feeding. Planaria (1.0-1.5 cm long) were placed in 24 wells of the plate culture (one in each well) and 2 mL of different concentrations of 1%-0.05% (w/v) of HECAC and HEC solutions were added. The control group consisted of 1% (w/v) BAC; an ingredient found in many mouthwashes. All the test substances were dissolved in APW. The planaria were kept in the plate in the dark at room temperature. The number of live/dead planaria was recorded after 24 hours. Planarians that did not move after gentle shaking were considered dead.

5.2.7.2.Planarian toxicity fluorescent assay

Planaria were treated with 1% (w/v) of the test substances for 24 hours, followed by a 0.1% (w/v) fluorescein solution in APW for 1 minute. Subsequently, the excess fluorescein solution was removed by washing the planarians in APW for 15 minutes. The planaria were placed on the slide and a few drops of 2% (w/v) agarose solution were placed on ice for immobilisation. Leica MZ10F stereomicroscope (Leica Microsystems Ltd., Wholesaler, UK) equipped with DFC3000G digital camera at 2.0× magnification, 160 ms exposure duration, and gamma 0.7 were used to record fluorescence images of the worms. The permeation of sodium fluorescein into the worms was evaluated using ImageJ software (version 1.8.0_112).

5.2.8. Preparation of films

1% (w/v) of HEC and HECAC polymers were prepared as solutions in DIW. Subsequently, 5mL of the prepared polymer solutions were poured into a plastic petri dish (40 mm diameter)

and stored at 30°C until dried (approximately 1-2 days). The films were cut out with a 12 mm diameter mould and stored in a dry place.

5.2.9. Scanning electron microscopy (SEM)

The surface morphology of all films was characterised by scanning electron microscopy (SEM). These techniques were also used to measure the thickness of the films. The film was mounted on an aluminium stud and secured with double-sided adhesive carbon tape. All films were coated with gold–palladium. The images were recorded at HT-15 kV accelerating voltage employing FEI Quanta 600 FEG SEM (FEI Company, Czech Republic).

5.2.10. Rheology

A modified version of the viscosity tests was used to determine the increase in viscosity by a mucin-polymer combination [15,16]. 1% (v/v) polymer stock solutions were prepared by dissolving the polymer in artificial saliva. The porcine mucin was obtained from the gastric mucosa of pigs and the 1% (v/v) mucin stock solution was prepared on the same day that the measurements were performed. A mixture of one-part mucin stock solution and one-part polymer solution was rapidly swirled together rapidly for fifteen minutes before performing the test. The ratio of mucin to polymer is 1 :1 (1 ml mucin stock solution was mixed with 1 ml polymer stock solution) according to a method by Shirit et al [8].

The tests were conducted using a Kinexus Malvern Rheometer, model KNX2100, equipped with a 25 mm cylindrical probe. The temperature was set at 37°C, and before the beginning of each measurement, the rheometer's temperature will be equilibrated. Shear rates ranged from 0 to 300 s^{1}, and three measurements were taken for each scan.

5.2.11. *Ex vivo* mucoadhesion study of HECAC films

The method was slightly modified following several studies [17–19]. The TA-XT Plus Texture analyser (Stable Micro Systems Ltd, Surrey, UK) with a 5 kg load cell was used to study the mucoadhesion profile of HEC and HECAC polymers. Sheep buccal tissue was cut into squares and placed between a cylindrical device and the top cover. The cover had a circular opening with a diameter of 20 mm. The mucosal surface of the tissue is exposed through the opening. Upon testing, the device and tissues were immersed in a 37°C water bath.

The films were attached to the 12 mm diameter aluminium probe with sticky adhesive tape and were lowered to contact exposed tissue. The test parameter settings were slightly modified and applied: pre-speed test 0.5 mm/s; test speed 0.5 mm/s; post-test speed 1.0 mm/s; applied

force 100g; contact time 30 s; trigger type auto; trigger force auto; and return distance of 20.0 mm. A force versus distance graph will be plotted to determine the maximum force and total work of adhesion.

5.2.12. Statistical analysis

A two-tailed student t-test with a 95% confidence interval as the minimal level of significance was employed as the statistical tool to evaluate the data.

5.3. Results

5.3.1. ^{1}H NMR spectra

We confirmed the chemical structure of modified HECAC using ^{1}H NMR presented in Figure 5.1. The presence of acryloyl groups was confirmed by the presence of new signals between 6.00 and 6.70 ppm, thus verifying the successful synthesis of HECAC. The signals between 6.00 and 6.70 ppm in the spectra of HECAC are generally similar to the ^{1}H NMR characterisation of acrylated PDMAEMA and acrylated chitosan reported previously [8,11]. During the synthesis, HEC was dissolved in TFA, resulting in the formation of some trifluoroacetyl ester groups on the hydroxyl groups of an anhydroglucose [20]. These acetyl protons give the signal at $\delta = 1.8$ ppm.

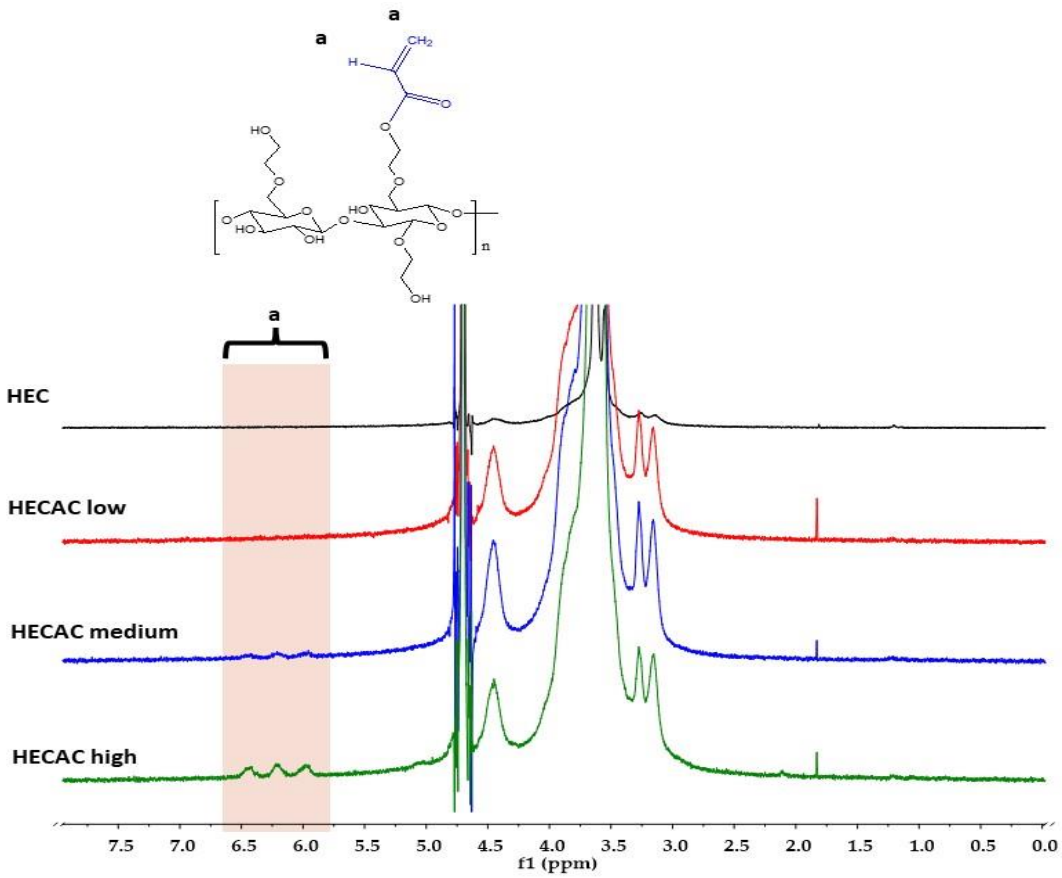


Figure 5.1 Structure and ^{1}H NMR spectra of unmodified HEC and modified HECAC polymers

5.3.2. FTIR spectra

Figure 5.2 shows the infrared absorption spectra for unmodified HEC and modified HECAC polymers. The successful modification of HECAC was confirmed by the appearance of a few new bands in the spectra shown in Figure 5.2. The FTIR-spectra of HECAC show the presence of a new band at 1610 cm^{-1} indicating the presence of carbonyl groups C=O is a specific signal for acryloyl in HECAC. Additional new bands at 1295 cm^{-1}, 1153 cm^{-1} and 1090 cm^{-1} correspond to C-O stretching of ester groups. While fingerprinting band at 714 cm^{-1} and 694 cm^{-1} shows C-H out of plane bending.

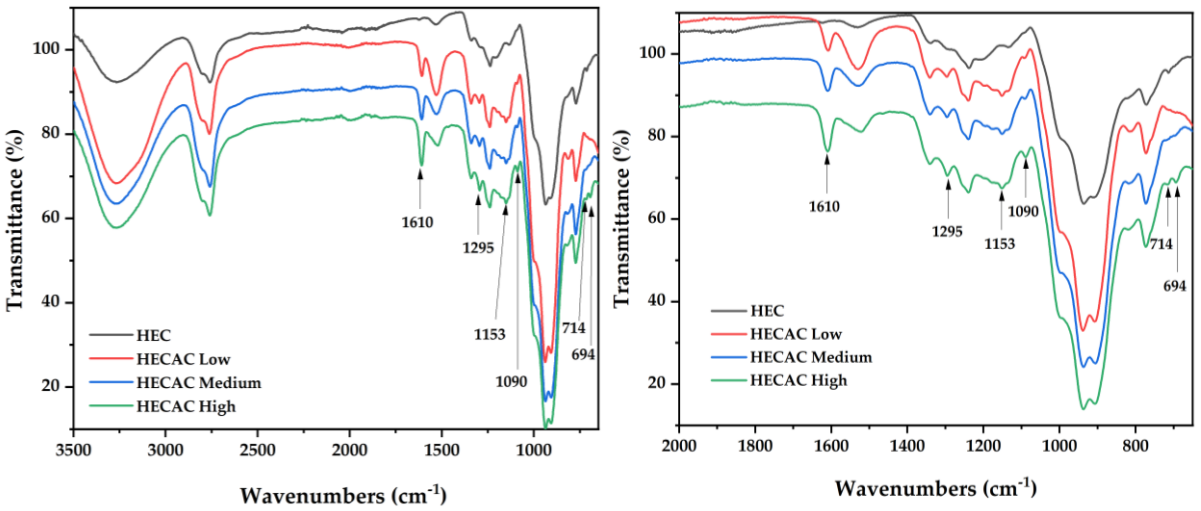


Figure 5.2. (a) FTIR absorption spectra of unmodified HEC and modified HECAC polymers at a different molar ratio (b) Zoom in of FTIR absorption spectra at 600-2000 wavenumbers (cm^{-1})

5.3.3. HPLC

To quantify the acryloyl groups in HECAC, samples were hydrolysed to acrylic acid. A linear correlation with a regression coefficient R^2 of 0.9996 and a linear equation of y = 171.88x + 48.661 was obtained for calibration (see Figure 5.3). The acrylic acid peaks detected had a retention time of 18–21 minutes.

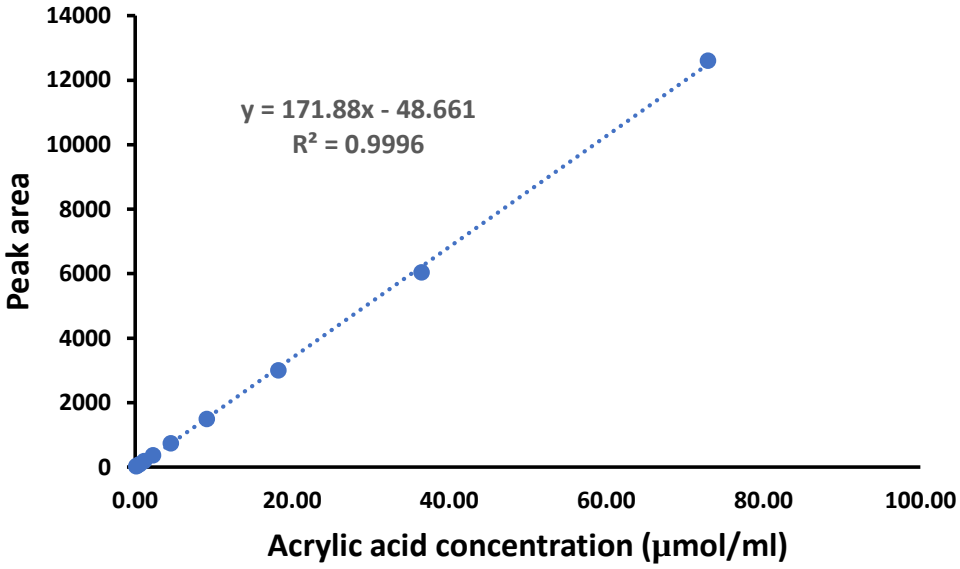


Figure 5.3. Standard calibration curve for acrylic acid

From calculation as shown in Table 5.1, the amount of acryloyl group was determined to be 191.06 ± -7.31 μmol/g HECAC low, 273.78 ± 6.21 μmol/g for HECAC medium and 379.38± 56.14μmol/g for HECAC high. In native HEC, no acrylic acid peak was found indication zero amount of acrylic acid in the samples.

Table 5.1. Calculation of amount of acrylic acid in HECAC samples

Sample	Peak	Concentration of acrylic acid (μmol/mL)	Actual amount of acrylic acid (μmol/g)
HEC	0.00	0.00	0.00
HECAC low	82.70 ± 14.26	0.76 ± 0.36	191.06 ± -7.31
HECAC medium	139.57 ± 7.76	1.10 ± 0.33	273.78 ± 6.21
HECAC high	212.17 ± 3.60	1.52 ± 0.32	379.38± 56.14

5.3.4. *In vitro* MTT toxicity assay

MTT toxicity assay was performed to assess the viability of cells in the presence of HEC and HECAC 24 hours after culture. According to Figure 5.4, cell viability was relatively significantly different between HECAC and HEC at all concentrations, especially for HECAC medium and HECAC high (p-value < 0.05). The viability of cells is less in HECAC solutions with the increase in concentration. However, cell viability for all polymers is above 50% after 24 hours, indicating that there is no toxicity.

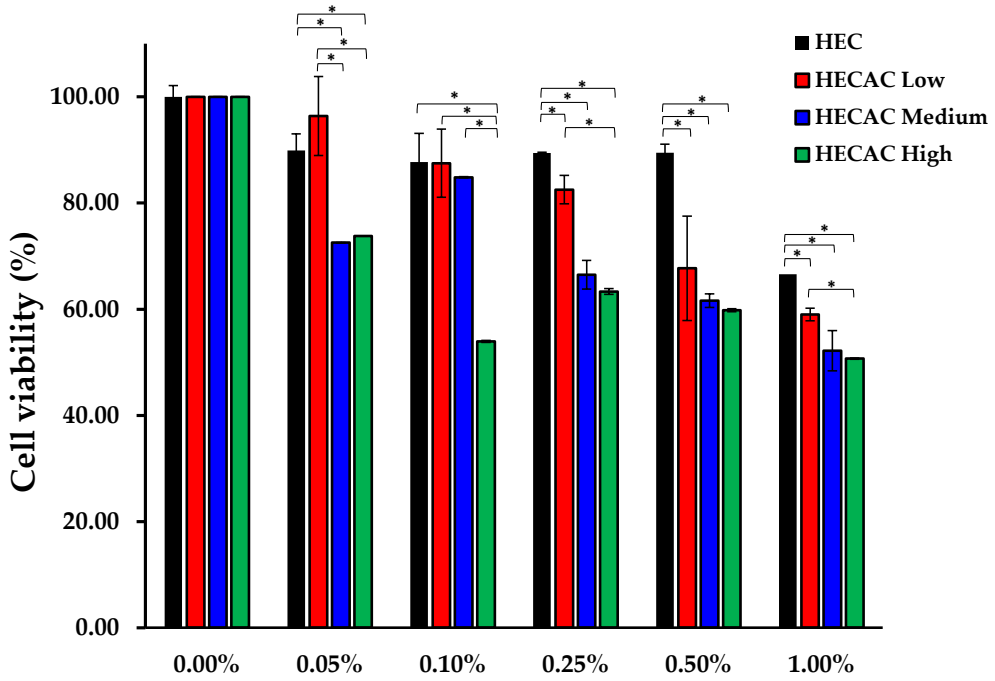


Figure 5.4. Caco-2 cell viability using MTT assay following their exposure to HEC and HECAC samples for 24 hours. Data show the mean ± standard deviations (n = 3). *Statistical significance was determined using t-test and $p < 0.05$ was statistically significant

5.3.5. *In vivo* planarian acute toxicity assay and fluorescent toxicity assay

Planarians have been used for toxicological screening of chemicals because their body wall is highly permeable and able to absorb low molecular weight compounds from their environment [21]. The results from the planaria dead and alive assay show that 1% (w/v) modified HECAC and 1% (w/v) HEC were not lethal to planaria after 24 hours.

To further evaluate the polymer absorption through the planaria's body wall we performed a fluorescent assay with live planaria. Fluorescent assays were conducted at 24 hours for 1% (w/v) working concentration of HEC and modified HECAC polymers. The results of the fluorescent assay are shown in Figure 5.4. From Figure 5.5, results show that only 1% (w/v) HEC and 1% (w/v) HECAC high exhibit a high fluorescence intensity with 1% (w/v) HECAC high has the highest value of 0.006 (a.u) while 1% (w/v) HEC has a value of 0.003 (a.u). Although 1% (w/v) HECAC high shows a significant increase in fluorescence intensity, it does not cause the death of the planaria. Upon comparison with positive control BAC (common ingredient in mouthwash), a 1% (w/v) of the positive control causes death to planaria in less than 1 hour (data not shown here).

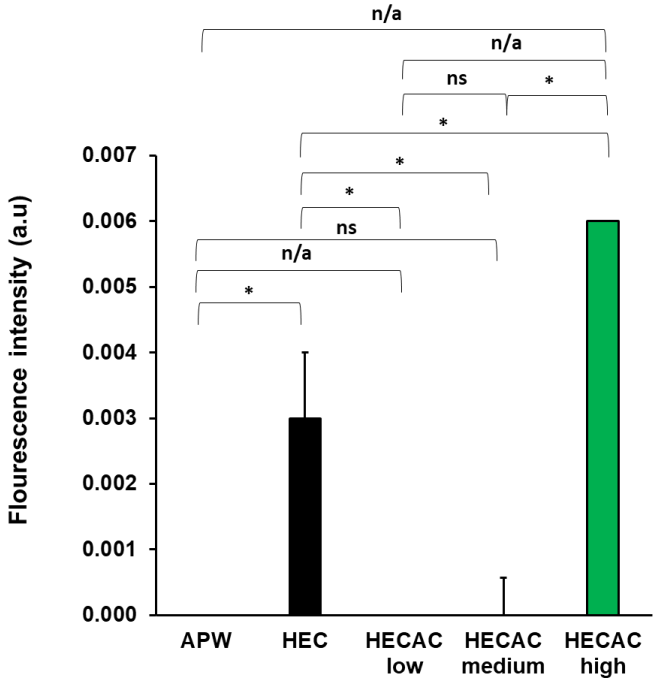


Figure 5.5. Fluorescent assay using planaria. Histograms represent the intensity of fluorescence in planaria following 24 hours of exposure to 1% (w/v) of HEC, HECAC low, HECAC medium and HECAC high. Data show the mean ±SE (n = 3). *Statistically significant according to t-test; $p < 0.05$

5.3.6. Physical characterisation of the films

The films were light, thin and clear with a soft and smooth texture. All the formulations, as shown in Figure 5.6 were easily removed from the mould.

Figure 5.6. Physical morphology of thin films at various concentrations from left to right HEC, HECAC low, HECAC medium and HECAC high

5.3.7. SEM

The average size of the film is 12mm in diameter and 50-80µm thickness in size as measured using SEM in Figure 5.7. It is suggested that the suitable thickness of the buccal films is in the range of 50 to 1000 µm [22]. The mentioned thickness of the film is without loading any drugs. A thin polymer film of 2-8 cm^2 area and 20-500 µm thickness can load active pharmaceutical ingredients (API) of less than 50 mg [23]. The developed blank film in all formulations is thus suitable to be placed in the mouth cavity and used for buccal drug delivery.

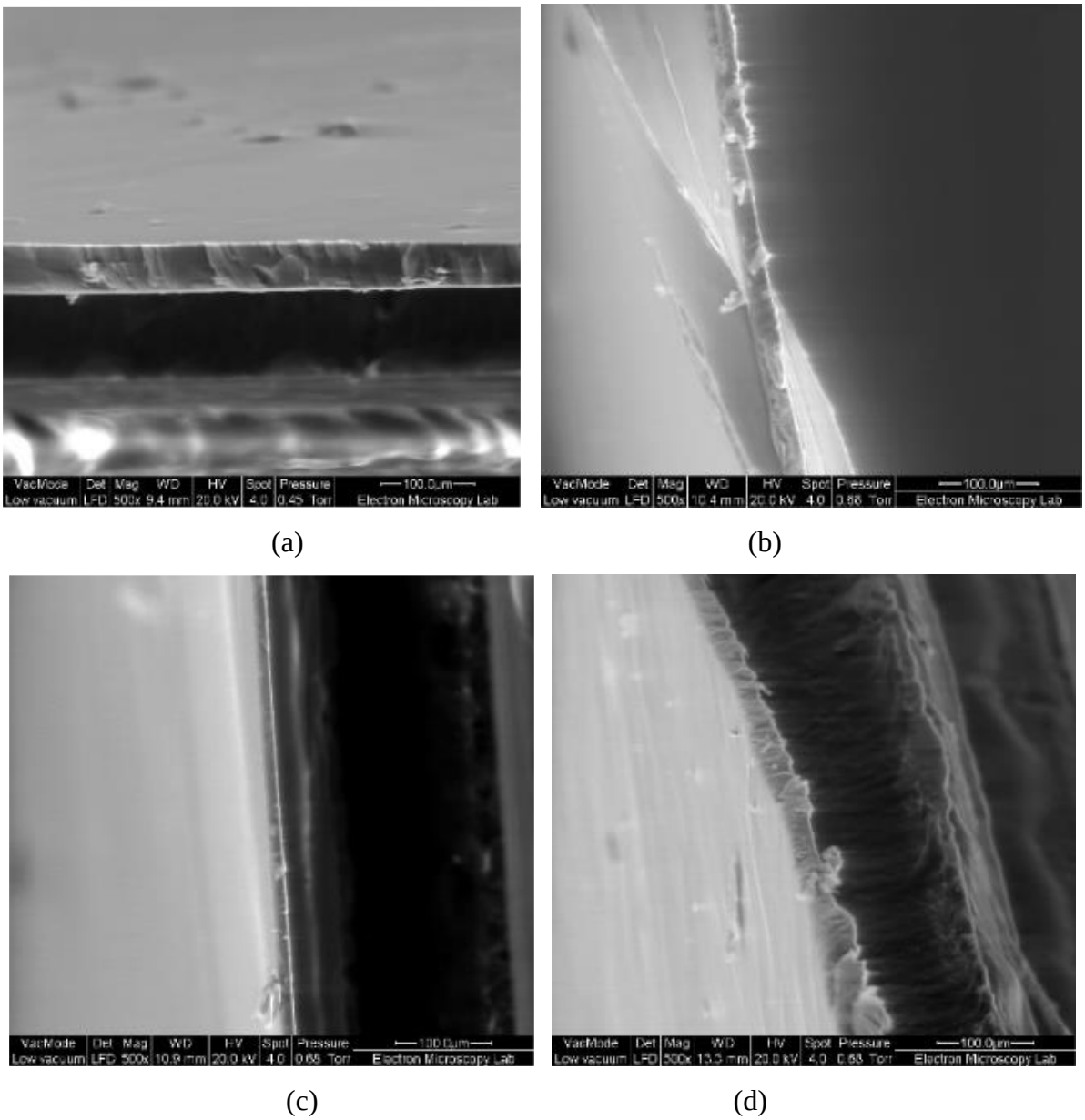
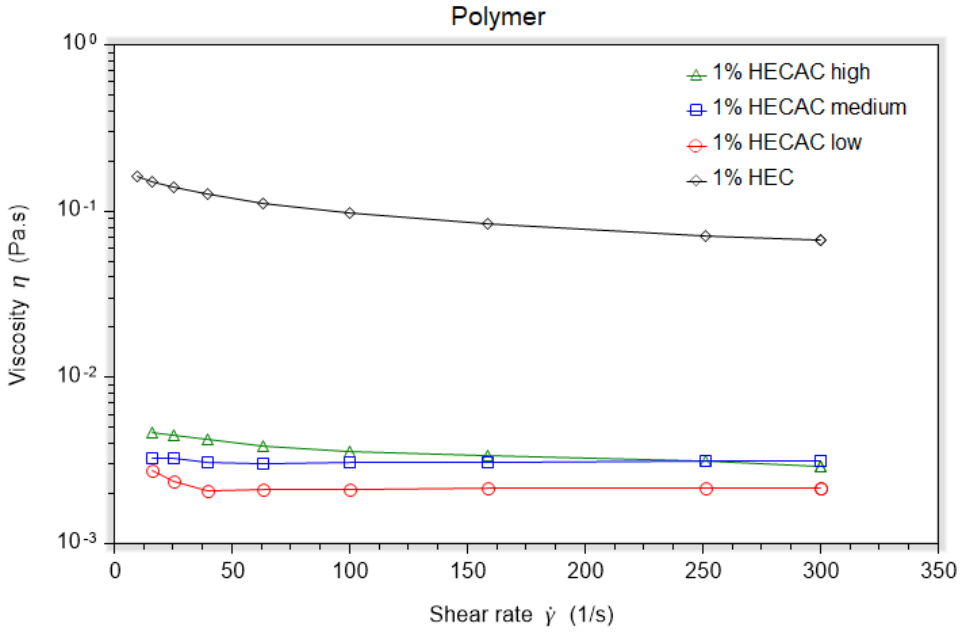


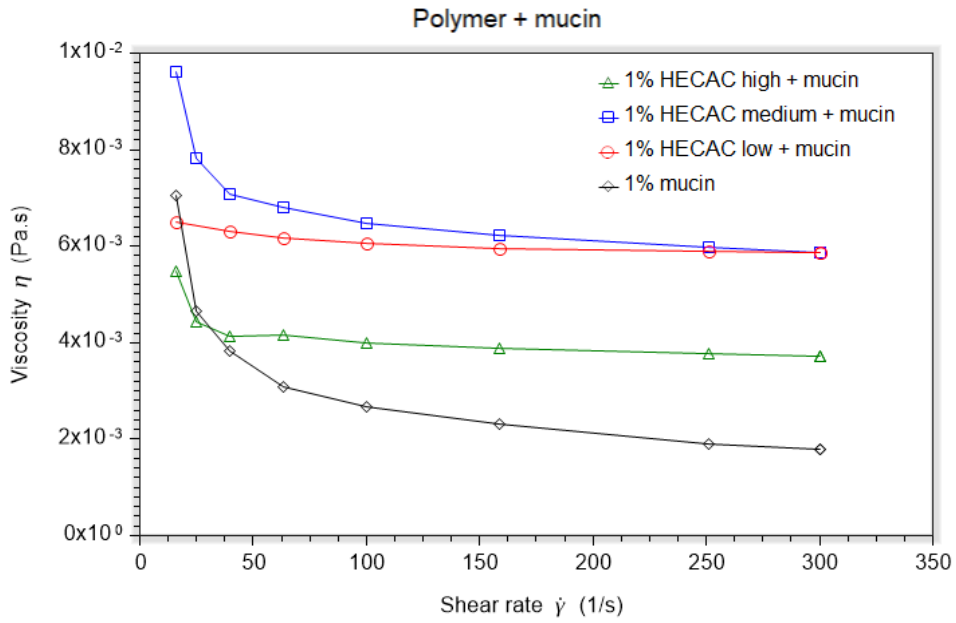
Figure 5.7. SEM images of thin films at various concentrations from left to right (a) unmodified HEC (b) HECAC low (c) HECAC medium and (d) HECAC high

5.3.8. Rheology

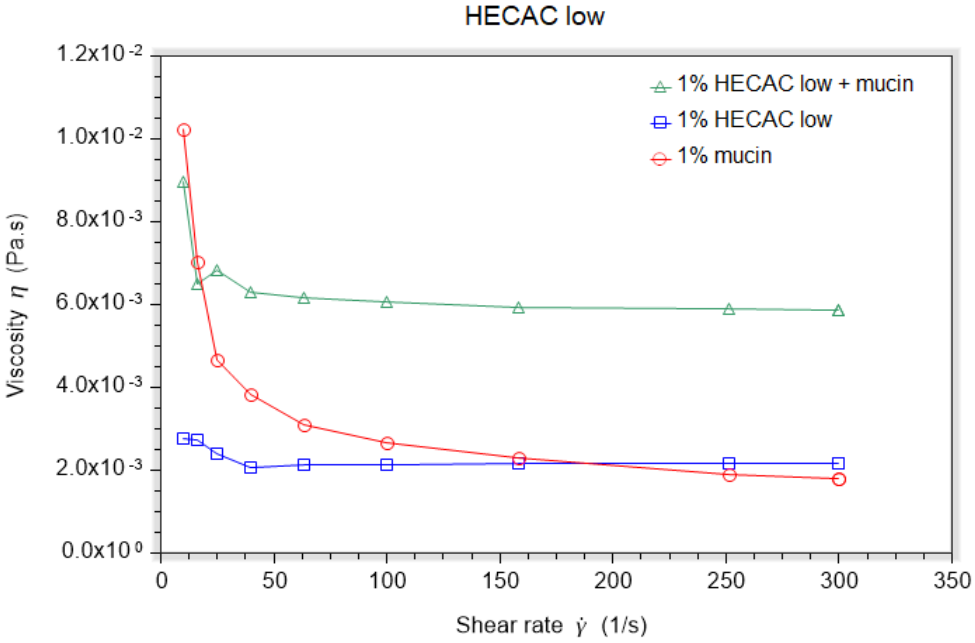
In this study, we modified the viscosity experiments described by Hassan and Gallo, in which viscometry variations in the system of mucosal tissue and selected polymers in solution were observed for the interaction between polymers and mucin [16]. Results from Figure 7 show all mixtures of HECAC and HEC with mucin show positive synergism, with the viscosity of the mixture of polymer + mucin being higher than the pure polymer. Figure 5.8(a) shows that HECAC high (triangle), HECAC medium (square) and HECAC low (circle) as a single polymer were less viscous than 1% (w/v) HEC (diamond) with the highest viscosity of 0.160 Pa·s at shear rate10.00 1/s. However, the value of viscosity of all polymers increases upon mixing with mucin. For modified HECVS polymers, the mixture of HECAC medium + mucin in Figure 5.8(b) has the highest viscosity 0.00962 Pa·s, followed by 0.00649 Pa·s for HECAC low + mucin and 0.00547 Pa·s for HECAC high + mucin at the lowest shear rate 15.85 1/s when compared to 1% (w/v) mucin at the same shear rate. Although the result of the mucin+ polymer mixtures show a positive synergism, an important finding of this rheology study is the force of bioadhesion could not be determined because the value was too low, possibly due to the low concentration of polymers and mucin.



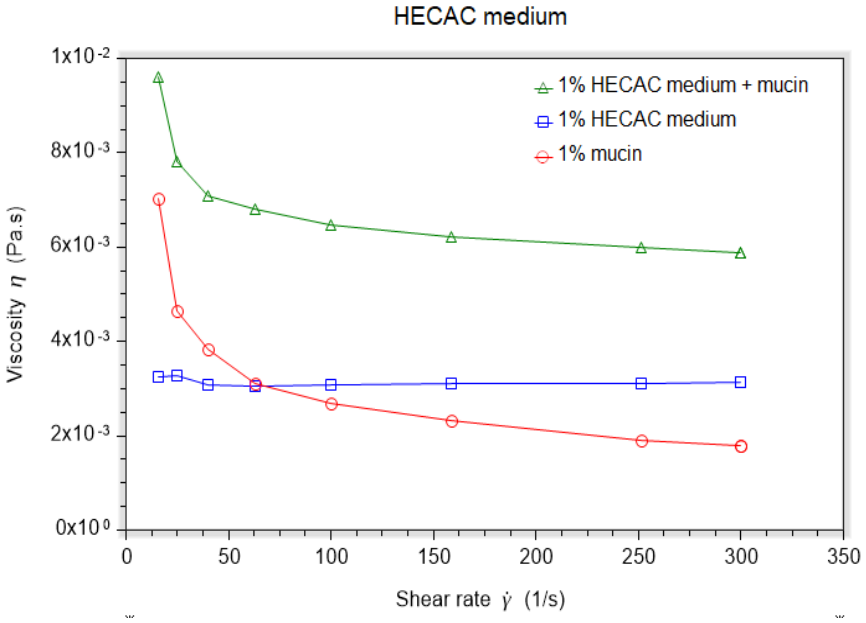
(a)


Polymer + mucin
1% HECAC high + mucin
1% HECAC medium + mucin
1% HECAC low + mucin
1% mucin
Viscosity η (Pa.s)
Shear rate γ̇ (1/s)
1x10-2
8x10-3
6x10-3
4x10-3
2x10-3
0x100
0
50
100
150
200
250
300
350

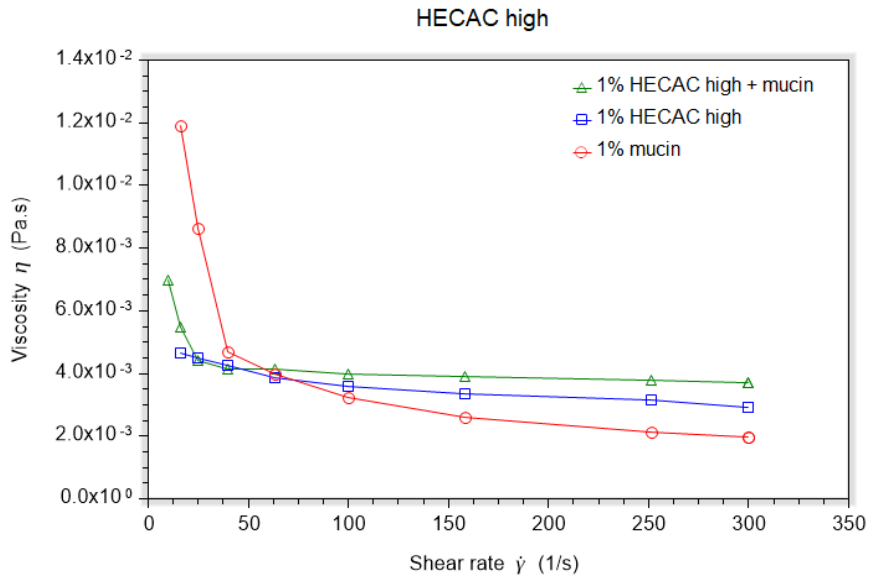
(b)


HECAC low
1% HECAC low + mucin
1% HECAC low
1% mucin
Viscosity η (Pa.s)
Shear rate γ̇ (1/s)
1.2x10-2
1.0x10-2
8.0x10-3
6.0x10-3
4.0x10-3
2.0x10-3
0.0x100
0
50
100
150
200
250
300
350

(c)



(d)



€

Figure 5.8. The rheology profile of (a) HECAC high, HECAC medium, HECAC low and HEC only (b) HECAC high + mucin, HECAC medium + mucin and HECAC low + mucin mixture (c) HECAC low (d) HECAC medium (e) HECAC high

5.3.9. Mucoadhesion profile

The results of the mucoadhesion studies show a significant improvement in the mucoadhesive properties of HECAC compared to HEC, as shown in Figure 5.9. The maximum detachment force and total work of adhesion of HEC compared to HECAC polymers were markedly improved. However, the best mucoadhesive properties were observed with HECAC medium.

HECAC low has the lowest mucoadhesive properties and HECAC high surprisingly has lower mucoadhesive properties than the modified HECAC medium.

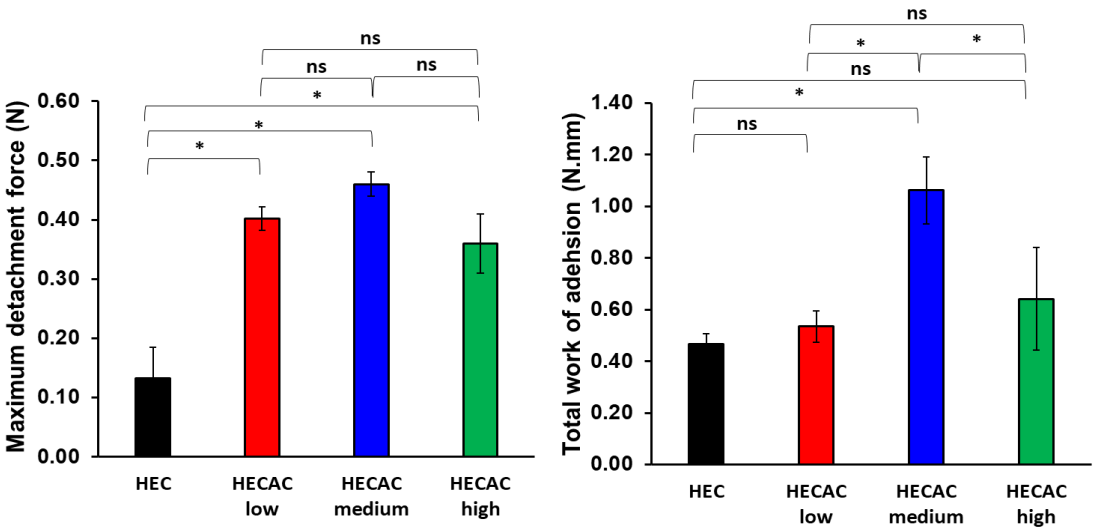


Figure 5.9. The mucoadhesion profile of unmodified HEC, HECAC low, HECAC medium and HECAC high (a) Maximum detachment force (N) and (b) Total work of adhesion (mm.N). Data showed the mean ±SE (n = 3) and * statistically significant according to t-test; $p < 0.05$

5.4. Discussion

The mucoadhesiveness of a dosage form depends on several factors, including the composition of the mucosal tissue and the physicochemical properties of the polymer formulation [3]. The formulation must be designed to produce specific interactions with mucin lining oral mucosal membranes [24]. The cysteine domains located in the central protein of the mucin glycoprotein are of particular interest to our research. In this study, we are inspired by the theory of sulfhydryl-acrylate interaction between the unsaturated groups in the polymer and the thiol group of the mucin glycoprotein via Michael addition that creates a strong mucoadhesive bond [6]. Thus, we aim to synthesis a poorly mucoadhesive, non-ionic HEC with an acryloyl group to improve the mucoadhesive properties of HEC.

In this study, acryloyl chloride was used to conjugate with HEC. Acryloyl chloride is the preferred chemical for conjugation of polymers with acryloyl groups in most studies [25]. In our experiments, we found that acryloyl chloride reacts exothermically and vigorously with water to form acrylic acid (data not shown here), and the synthesis is ineffective. Most work with acryloyl chloride uses organic solvents for the synthesis, which is a limitation for HEC as it dissolved in water. In this work, we homogeneously dissolved HEC in trifluoroacetic acid.

Trifluoroacetic acid is a non-aqueous solvent used to dissolve cellulose [26]. However, the solvent reacts chemically with HEC by trifluoroacetylated selectively in the C6 hydroxyl groups, as seen in the ^{1}H NMR spectra in Figure 5.1 at a signal of 1.8 ppm [20]. We verified the successful synthesis of HECAC by FTIR and quantified the acryloyl group by HPLC. The new signals at 6.00 and 6.70 ppm from HPLC and band confirmed this.

The planaria assay is an attempt to find a lower order animal model that can be used alternatively for acute toxicity testing for humans. Planarians in particular are easy to keep in the laboratory and are sensitive to environmental toxins [27]. We use sodium fluorescein because it is a safe dye that can penetrate injured tissue, and this property has been used to determine the extent of injury. This test has proven useful in screening neurotoxic and dermal irritant compounds [28,29]. When comparing the two toxicity assays, we found a correlation between the assays. Both toxicity tests confirmed that the 1% (w/v) working concentrations of HEC and HECAC are safe for human use.

Mucoadhesive interactions between mucin and polymers have a complex nature. Referring to Figure 5.7 the synergism observed in the rheology test according to Hassan and Gallo was attributed to physical chain entanglement and non-covalent bonds (hydrogen, van der Waals, etc between mucin and polymer [16]. We anticipated the synergism was additionally contributed by the covalent bonding of double bonds in acryloylated HEC and cysteine in the mucin glycoprotein. However, the results from rheology did not match the results from the tensile test. It has been reported in a few studies that tensile strength and viscosity do not correlate to study mucoadhesion [15,30,31]. This is contradicted by reports in several publications that find a correlation between rheology and tensile testing [32,33].

HEC was found to have the highest viscosity as probably additional physical entanglement and secondary chemical bonding strengthen the network [34]. The results of viscosity ranking was HEC > HECAC medium > HECAC low > HECAC high. This behaviour could be because modification with an acryloyl group leading to less flexibility and mobility of the polymer chain with high molar ratio modification. This pattern was also observed in polyacrylates with similar molecular weight and different degrees of alkylation [35,36]. Therefore, further rheological experiments with various polymer concentrations and mucin: polymer weight ratio as well as molecular characterization using ^{1}H NMR and AFM were suggested for further investigation of the interaction between acryloyl groups and mucin.

In this study, the force of bioadhesion was not determined because the value was too low, which could be due to the low concentration of polymer and mucin. If the mucin concentration is too low, the interaction between polymer and mucin is unstable, while a high concentration

results in the polymer network being impermeable to the solvent, so that the free polymer chains cannot diffuse into the mucosal surface [37]. This was also agreed by Madsen et al, that rheological synergism only occurs within a certain concentration range of the polymer depending on the material [34]. Therefore, further work should add the different concentration of polymer and mucin as a factor to investigate the optimal mucoadhesion interaction.

The mucoadhesion results from the tensile test of modified HECAC showed the highest adhesion properties at the highest molar ratio. The ranking was HECAC medium > HECAC high > HECAC low > HEC. The ranking was not in agreement with previous studies on modified polymers with acryloyl groups [35,38]. This might have resulted from the chain entanglement from the interaction of HECAC polymers with the surface of mucin by covalent and non-covalent bonds as well as hydrogen bonds. An increase in acrylate content could be expected to increase the probability of chain entanglement.

5.5. Conclusions

We have demonstrated in this study that the mucoadhesive properties of non-ionic HEC undergo a significant improvement after being chemically modified with acryloyl groups. The ^{1}H NMR and FTIR spectroscopy verified the successful introduction of acryloyl groups into HEC, while HPLC was used to determine the amount of the acryloyl group. We performed a simple rheology study measuring viscosity at a different shear rate rather than viscoelasticity. We have found the ranking order of components of viscosity of mucin with polymer is not similar to the tensile test. A positive synergism upon an increment of viscosity of polymers in excess of mucin is to be expected as this interaction will contribute to the high strength of polymers adhesion to mucosa tissue. Thus, we suggested performing more rheology work at different concentrations of polymer and mucin and molecular characterisation work to further study the interaction between mucin and polymers. Toxicity of HECAC polymers was not detected in the *in vitro* MTT assay or the *in vivo* planaria assay, suggesting that these polymers are completely non-hazardous. The outcome of this study supports the idea that HECAC could be used as a new excipient for a transmucosal drug delivery system with improved mucoadhesive properties.

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Chapter 6.

Vinyl sulfone functionalised hydroxyethyl cellulose as a new excipient with enhanced mucoadhesive properties.

(QUAD system contribution of Fhataheya Buang: 60% of conception and design, 100% of data collection, 70% of data analysis and conclusions, and 70% of manuscript preparation).

Chapter 6.

Vinyl sulfone functionalised hydroxyethyl cellulose as a new excipient with enhanced mucoadhesive properties

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Abstract

The development of mucoadhesive excipients is a strategy to improve drug delivery to the mucosal surface. These excipients have the ability to adhere to the mucosal surfaces and help prolong the delivery of the drug. It has been shown that modification of polymers with unsaturated groups can improve the mucoadhesion properties of the polymer. Therefore, in this study, a vinyl sulfone group was introduced into the structure of HEC in a simple one-pot synthesis method and the properties of the modified HEC were investigated. Using ^{1}H NMR and FTIR spectra, the successful synthesis of vinyl sulfone derivatives of HEC (HECVS) was confirmed. Elemental analysis was used for the quantification of vinyl sulfone. The planarians acute toxicity test has shown that the safe concentration for HECVS high is below 0.25% (w/v), HECVS medium is below 0.5% (w/v) and HECVS low is below 1% (w/v). 1% (w/v) of all HECVS polymers may cause irritation indicated by an increase in fluorescence intensity while *in vitro* MTT assay with Caco-2 cell shows no sign of toxicity. The results from a tensile test of HECVS microparticles showed an improvement in the mucoadhesive properties. The results demonstrate that HECVS improved the mucoadhesiveness of HEC and is safe to use at a lower modification molar ratio of DVS to HEC.

Keywords: hydroxyethyl cellulose; mucoadhesion; vinyl sulfone; oral delivery; microparticles

6.1. Introduction

Transmucosal delivery of drugs has several advantages over other methods of administration. Drugs administered via this route bypass hepatic first-pass metabolism and thus avoid degradation by gastrointestinal enzymes [1]. However, the mucus layer, which acts as a barrier and lubricates the mucosal tissue, may prevent the drugs from reaching the epithelial site, compromising drug absorption and therapeutic efficacy [2].

To prolong the residence time of the drug on the mucosal surface, the excipients must have good mucoadhesive properties. Unfortunately, not all polymers are mucoadhesive. Good mucoadhesive polymers are polymers that have (i) strong hydrogen bonding groups, e.g. carboxyl, hydroxyl, amino and sulphate groups, (ii) strong anionic or cationic charges, (iii) high molecular weight, (iv) chain flexibility, (v) surface energy properties that favour spreading on mucus [3].

In 2010, Professor Bianco-Peled proposed that a polymer bearing an acrylate end group can interact with the sulfide end group of the mucin-type glycoprotein by a Michael-type addition reaction [4]. The thiol residue on the mucin glycoprotein backbone, acting as a strong nucleophile, attacks the double bond of acrylate and a covalent bond is formed while hydroxyl is released. This interaction increases the adhesive force between the polymer and the mucus.

Discovered in 1880 by Arthur Michael, the Michael addition reaction is the addition of stabilized anions to α,β-unsaturated carbonyl and related compounds [5]. This reaction is a simple, robust and highly effective reaction under relatively easy reaction conditions [6]. Divinyl sulfone molecule contains two electrophilic vinyl groups and it is a good Michael acceptor including other electron-deficient unsaturated groups such as methacrylates, and maleimides [7–9].

The cross-linking of DVS with the hydroxyl group of a polysaccharide is of great interest, mainly because the interaction creates a gel network [10,11]. However, such cross-linking work results in a product that is not water-soluble and not mucoadhesive [12]. According to Yu et al, a polymer has low solubility when the DVS/OH ratio is high. Yu et al. also suggested that the available quantity of nucleophilic alkoxide ions and the amount of DVS are essential for the preparation of a water-soluble polymer [13].

Previous work has shown that HEC has good cross-linking efficiency with DVS due to the reactivity of the hydroxyl groups with less steric hindrance [14]. This leads to effective modification of HEC with divinyl sulfone, which, however, tends to form a hydrogel network. Nevertheless, there have been numerous successful attempts to conjugate cellulose with vinyl sulfone. For example, vinyl sulfone modified with the sodium salt of hyaluronic acid (HA) was

synthesised by using the tetra-n-butylammonium salt of HA with vinyl sulfone cysteamine [15]. Another work recently reported is the vinyl sulfone modified with silk, in which under basic conditions and in the presence of the electrophile DVS, the nucleophilic alkoxides and primary amines reacted with the silk to form the conjugated polymer [16].

Therefore, we attempt to conjugate HEC with DVS in a one-pot synthesis procedure to prepare HEC vinyl sulfone esters with an ethyl spacer between the thioether and ester groups following a method developed by Hiemstra et al [17].

6.2. Materials and methods

6.2.1. Materials

HEC 720,000 Da, triethylamine (TEA), divinyl sulfone (DVS), chitosan low molecular weight (50,000-190,000 Da, 75-85% deacetylated), chitosan medium molecular weight (190,000-310,000 Da, 75-85% deacetylated), chitosan high molecular weight (310,000-375,000 Da, >75% deacetylated) dimethylsulfoxide (DMSO), dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), hydrated p-toluenesulfonic acid (PTSA) 3-Mercaptopropionic acid (3-MPA), sodium hydroxide were purchased from Sigma Aldrich Co., Ltd., Gillingham, UK. All reagents used in the study were of analytical reagent grade.

DPTS was prepared according to Hiemstra et al from 4-(dimethylamino)pyridine (DMAP) and hydrated p-toluenesulfonic acid (PTSA) and recrystallised from toluene [17].

The cell culture materials DMEM High Glucose (Capricorn Scientific GMbH, Germany), fetal calf serum (GE Healthcare Life Sciences, Chicago, IL, USA), penicillin/streptomycin (Nacalai Tesque Inc., Kyoto, Japan), CellTiter 96 Aqueous MTS reagent powder (Promega Corporation, Winsconsin, USA) and phenazine methosulfate (Thermo Fisher Scientific, Leicestershire, UK), was used for cell viability assay. Caco-2 cells were donated by the Faculty of Pharmacy, University Teknologi Malaysia, Puncak Alam, Malaysia.

Excised sheep upper and lower lips were obtained from a local abattoir PC Turner Abattoir (Farnborough, Hampshire, UK).

6.2.2. Synthesis of HECVS

HECVS were synthesised at room temperature using a one-pot method slightly modified from Hiemstra et al [17]. The HECVS polymers were synthesised by forming a vinyl sulfone ester with an ethyl spacer between the thioether and the ester groups. Different molar ratios of 3-MPA to HEC were used to achieve different degrees of substitution (DS) as shown in Table

6.1. In the initial step, 3-MPA was added dropwise to 90 mL of dissolved DVS in DMSO, and the reaction was agitated at room temperature (25°C) for 4 hours.

1% (w/v) of HEC, DPTS and DCC were dissolved in 60mL DMSO and added to the DVS/3-MPA mixture and continued stirring for a further 24 hours. This process produces N,N-dicyclohexylurea (DCU) salt, which is recovered by precipitation in cold ethanol after filtering off. The precipitate was purified by ultrafiltration after washing with ethanol and dissolving in distilled water. Details on the schematic synthesis reaction of HECVS can be found in Figure 6.1.

Table 6.1. Details on synthesis method of HECVS

ID	Percentage Polymer (%)	Molar Ratio [DVS]:[3-MPA]	Molar Ratio [HEC]:[3-MPA]	Molar Ratio [DPTS]:[3-MPA]	Molar Ratio [DCC]:[3-MPA]
HECVS low	1.0	[20]:[1]	[1]:[0.1]	[0.15]:[1]	[1.5]:[1]
HECVS medium	1.0	[20]:[1]	[1]:[0.3]	[0.15]:[1]	[1.5]:[1]
HECVS high	1.0	[20]:[1]	[1]:[0.9]	[0.15]:[1]	[1.5]:[1]

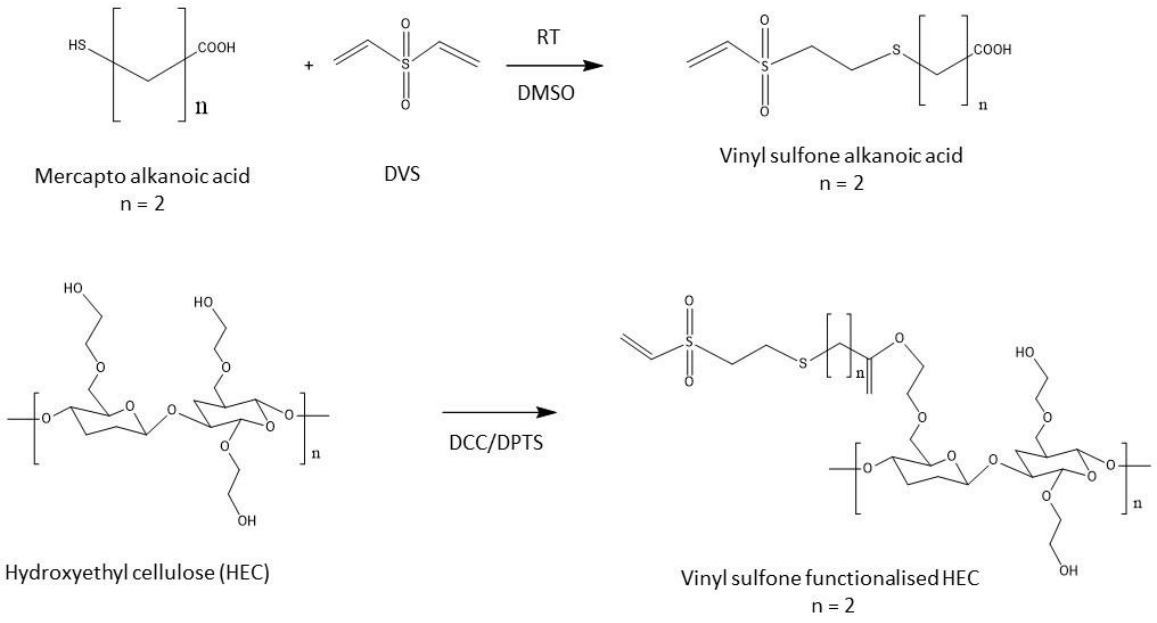


Figure 6.1. Schematic representation of synthesis procedure of HECVS polymers with ester spacer between the thioether and ester groups

6.2.3. $^{\cdot 1}$H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy

Solutions of modified HECVS were prepared in deuterated D_2O in 5 mm diameter NMR tubes. The ^{1}H NMR spectra were performed on a 400 MHz Ultrashield Plus™ B-ACS 60 spectrometer (Bruker UK Ltd., Coventry, UK.). Results were analysed using MestReNova (Mnova) software Version 6.0.2-5475 (Mestrelab Research, S.L., Spain).

6.2.4. Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectra of HEC and HECVS were recorded using a Spectrum 100 FTIR Spectrophotometer (Perkin–Elmer UK Ltd., Buckinghamshire, UK) from 4000 to 650 cm^{-1}, with a resolution of 4 cm^{-1}, and an accumulation of 16 scans. The data obtained were analysed using Spectrum One software (Perkin–Elmer UK Ltd., Buckinghampshire, UK).

6.2.5. Elemental analysis

Elemental analysis of HEC and modified HECVS was used to determine the degree of substitution (DS). An amount of 10mg each of HEC and HECVS polymers were dried in hot air oven overnight. Samples was sent to Medac Ltd, UK for analysis of the composition of sulfur in the samples. The DS was determined by the average number of sulfate groups attached to a glucose unit calculated according to Fan et al [18]. The DS of HECS was determined by using the following equation:

$$\frac{228.\,N\%}{3200-102.N\%} \qquad (1)$$

6.2.6. Planarian acute toxicity assay and fluorescent toxicity assay

Schmidtea mediterranea were donated by Oxford Brooks University and kept in artificial pond water (APW) at room temperature. They were fed chicken liver once a week and the APW was changed weekly. APW was prepared with different salt concentrations of 5 M NaCl, 1 M $CaCl_2$, 1 M Mg SO_4 and 1 M KCl dissolved in 50 mL ultra-pure water (UPW) as a stock solution and further diluted in 20 L of UPW. Planaria (1.0-1.5 cm long) were placed in 24 well plate cultures (one in a well) and 2 mL of different concentrations (0.05% w/v, 0.10% w/v, 0.25% w/v, 0.50% w/v and 1.00% w/v) of HECAC solutions and HEC were added. All test substances were dissolved in APW.

Planaria were kept in a plastic container in the dark at room temperature. The acute toxicity test was performed for 24 hours and the number of live/dead planarians was recorded. Planaria that stopped moving after gentle shaking of planaria/water were considered dead.

Following the acute toxicity test, Planaria were treated with 1% (w/v) of the HECVS and HEC for 24 hours and then placed in a 0.1% (w/v) fluorescein solution in APW for 1 minute. The excess fluorescein solution was then removed by washing the planaria in APW for 15 minutes. The planarians were placed on a glass slide and a few drops of 2% agarose solution were placed on ice and frozen for immobilisation. Leica MZ10F stereo microscope (Leica Microsystems Ltd., Wholesaler, UK) equipped with DFC3000G digital camera at 2.0× magnification, 160 ms exposure duration, and gamma 0.7 were used to capture fluorescence images of the worms. The permeation of sodium fluorescein into the worms was evaluated using ImageJ software (version 1.8.0_112).

6.2.7. *In vitro* toxicity

Caco-2 cells were used to determine the cytotoxicity of all polymers. Cells were cultured in DMEM High Glucose supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. They were kept in a 37 ºC incubator with 5 % CO^2 in the air and 100 % relative humidity.

Cell viability was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS assay). Cells were seeded in 96-well plates at a density of 3×10^3 cells/well and incubated overnight at 37 ºC in 5% CO_2 humidified air to allow cells to attach. The cells were then treated with different concentrations of the compounds for 24 hours. The negative control group consisted of untreated cells and was considered 100% viable cells. After 24 hours, 20 µL of the solution MTS, containing 2 mg/mL of CellTiter 96 Aqueous reagent powder MTS (Promega Corporation, USA) and 0.92 mg/mL phenazine methosulphate (Thermo Fisher Scientific, USA), was added to each well. The cells were incubated for 4 hours at 37 ºC in a humidified 5% CO_2 incubator. Absorbance (Abs) was measured at 490 nm using the Infinite 200 PRO microplate reader (Tecan Group Ltd., Switzerland).

6.2.8. Preparation of microparticles

HEC and HECVS were dissolved separately in deionised water, while chitosan (low, medium and high molecular weight) was dissolved in 0.1M HCL. Each solution was spray dried (Buchi Mini Spray Dryer B-290, Zurich, Switzerland) with the following parameters: inlet temperature of 120°C, pump setting of 0.10% and aspirator 100%

6.2.9. Scanning electron microscopy (SEM) of microparticles

The microparticles were characterised by scanning electron microscopy (SEM) to measure their size. All formulations were attached to an aluminium stud and secured with double-sided tape. It was then coated with gold palladium. Images were recorded at HT -15 kV accelerating voltage using FEI Quanta 600 FEG SEM (FEI Company, Czech Republic).

6.2.10. *Ex vivo* mucoadhesion study of HECVS microparticles

The TA-XT Plus Texture Analyser (Stable Micro Systems Ltd, UK) with a 5 kg load cell was used to investigate the mucoadhesion profile of all formulations listed in Table 6.1. Sheep buccal tissue was cut into squares and placed between a cylindrical device and the top cover. The cover had a circular opening with a diameter of 20 mm. The mucosal surface of the tissue is exposed through the opening. After the test, the device and the tissue are immersed in a 37°C water bath.

An adhesive tape was used to connect the wafers to the aluminium probe of 12 mm diameter and lowered until contact with the exposed tissue. The following test parameters were changed slightly: pre- speed before test 0.5 mm/s; test speed 0.5 mm/s; post- speed after test 1.0 mm/s; applied force 100g; contact time 30 s; trigger type auto; trigger force auto; and return distance 20.0 mm.

6.2.11. Statistical analysis

The statistical tool used was a two-tailed Student t-test using version 17 of SPSS. P-values < 0.05 were considered statistically significant.

6.3. Results

6.3.1. ^{1}H Nuclear magnetic resonance spectroscopy (^{1}H NMR) spectra

According to Figure 6.2, the signals at 6.4 ppm and 6.8 ppm (peaks d and e) were assigned to the vinyl sulfone proton in agreement with Hiemsestra et al [2]. The signals at 2.8-3.0 ppm (peaks a) were assigned to a (-CH_2-CH_2-S-CH_2-CH_2-) where n=2 referred to the structure scheme in Figure 6.1. However, due to the overlap of peaks a on the HEC backbone, it is impossible to calculate the degree of substitution from the spectra. D'Avino et al.(2022) and Ray et al.(2018) previously reported the peaks at 1.21 ppm and 1.83 ppm of unidentified structure present in HEC [19,20].

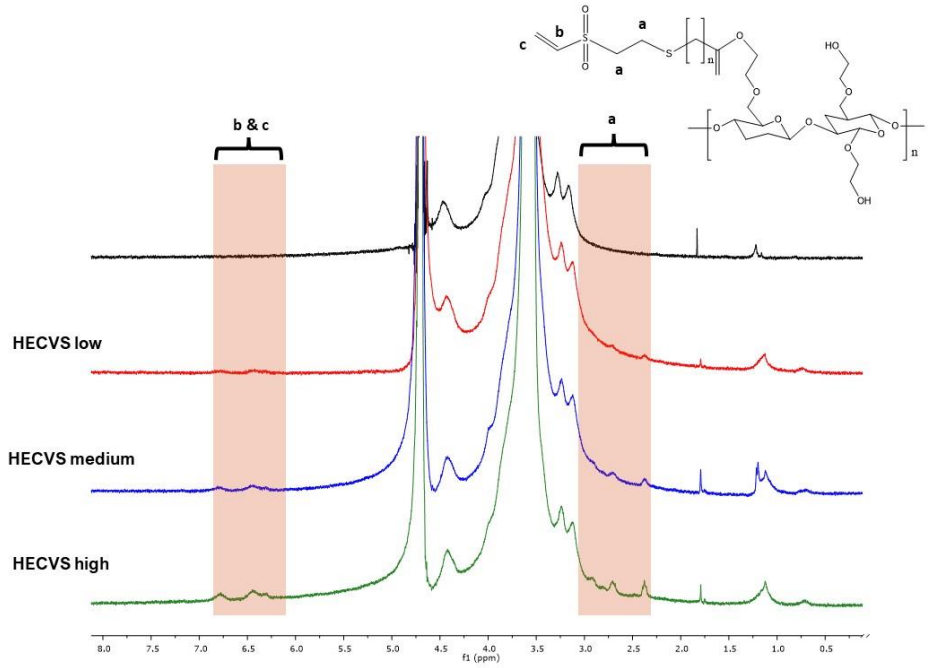


Figure 6.2. Structure and ^{1}H NMR spectra of unmodified HEC and modified HECVS polymers

6.3.2. Fourier transform infrared (FTIR) spectra

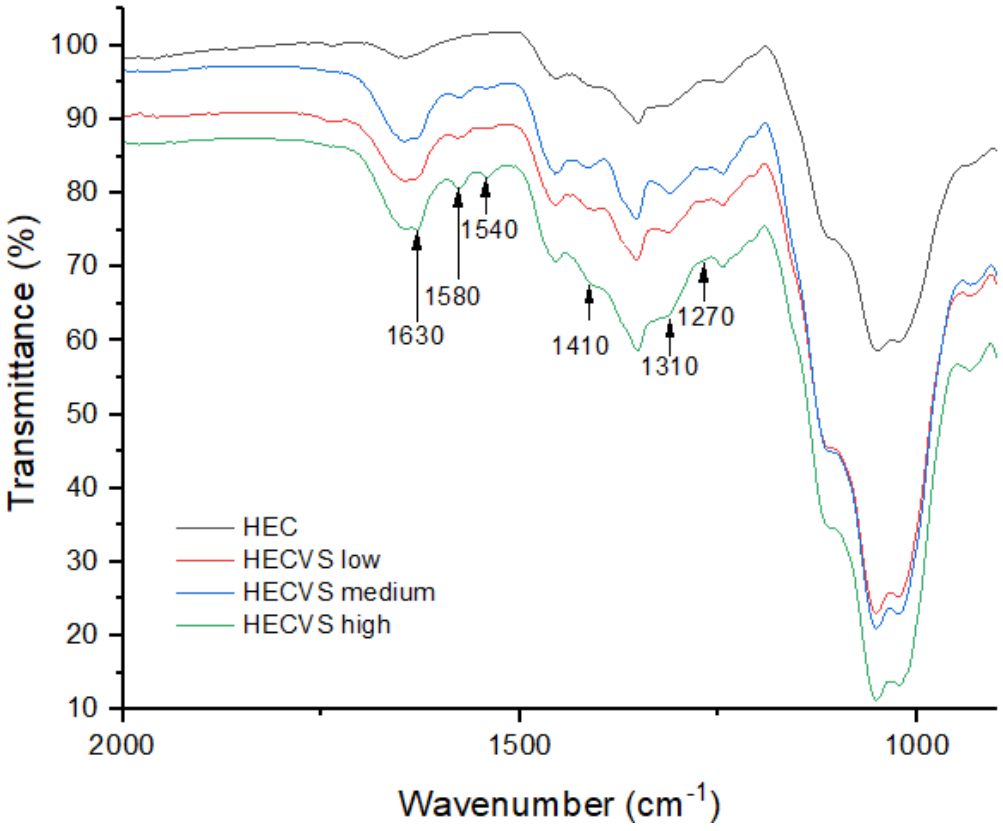


Figure 6.3. FTIR spectra of HEC and HECVS at various molar ratios

Figure 6.3 displays the FTIR spectra of HEC and HECVS with different molar ratios. For VS the most evident absorptions occur at 1310 cm^{-1} and 1270 cm^{-1} (S-O asymmetric and symmetric stretching vibrations) which exist in all HECVS spectra [21,22]. There is also a slight difference in the band intensity at ~1410 cm^{-1} (C=O asymmetric stretching vibrations (carboxylate groups) suggesting the existence of hydroxyl and carboxyl functional groups in HECVS. The Carboxylate group band appears at around 1630-1540 cm^{-1} with an increase in intensity in HECVS high compared to HECVS low and HECVS medium [21].

6.3.3. Elemental analysis

According to the elemental analysis, the sulfur content of the modified HECVS increased with increasing molar ratio of DVS to HEC. It is expected the larger the molar ratio, the higher the sulfur content in the polymer. Table 6.2 showed the amount of sulfur ranges from 1.04 ± 0.04 to 1.59 ± 0.01%, and the estimated DS range from 0.08 ± 0.08 to 0.12 ± 0.00. From the results, we can confirm that HECVS with varying degrees of substitution had been prepared successfully. The details on the calculation can be found in Appendix XII and Appendix XIII

Table 6.2: Sulfur content and DS of the HECVS with different molar ratio

	S%	DS_{EA*}
HEC	0.00	0.00
HECVS low	1.04 ± 0.04	0.08 ± 0.08
HECVS medium	1.21 ± 0.21	0.09 ± 0.01
HECVS high	1.59 ± 0.01	0.12 ± 0.00

*DS_{EA} calculated from elemental analysis

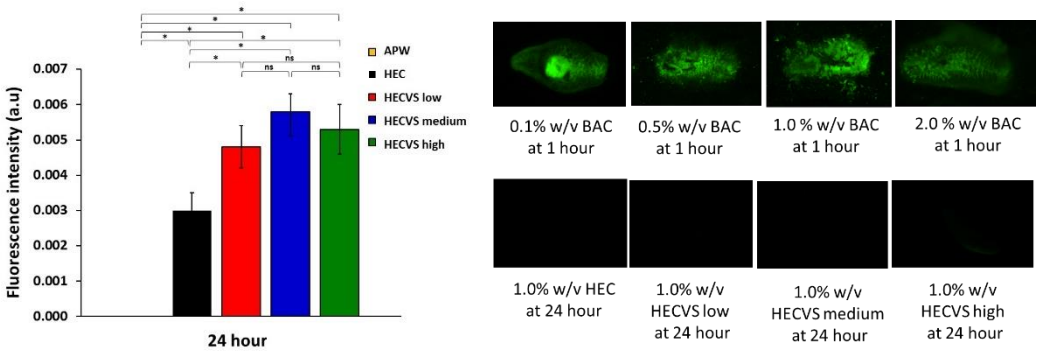
6.3.4. Planarian acute toxicity assay and fluorescent toxicity assay

HEC is a safe polymeric excipient as documented in the monographs approved by the Global Pharmacopoeia. However, after modification, the modified HEC, like other chemicals, is potentially toxic and must be proven safe before use. In this study, we tested the modified HECVS solutions with a new invertebrate model for toxicological screening with planarian worms.

DVS is known to be hazardous due to the reactivity of its vinyl groups[23]. In a study with rats, an intraperitoneal (IP) dose of 3.5 mg/kg vinyl sulfone resulted in 100% mortality with an average time to death of 5 days, while 2.5 mg/kg vinyl sulfone resulted in 40% mortality with an average time to death of 7 days. The intraperitoneal LD50 was calculated to be about 3 mg/kg [24].

In all planarian worms, 100% mortality was observed after 24 hours of interaction with 1.00% (w/v) and 0.5% (w/v) of HECVS high and 1.00% (w/v) of HECVS medium polymer solutions. We found that at a concentration below 0.25% (w/v) of HECVS high and below 0.5% of HECVS medium, no mortality was found. While for HECVS low, no mortality was recorded.

Following the acute toxicity assay, we performed an additional test (fluorescent assay) with the planarian worms. The fluorescent assay assesses the effect of substances on the integrity and function of the body wall of planarians [25]. If the integrity of the planarian body wall is disturbed, the fluorescent dye is absorbed and can be quantified under the fluorescence microscope.



(b) (b)

Figure 6.4. a) Fluorescent assay using planaria. Histograms represent the intensity of fluorescence on planaria after exposure to 1.0% (w/v) of APW, unmodified HEC, HECVS low, HECVS medium and HECVS high. (b) Fluorescent images of planaria exposed to various concentrations of BAC at 1 hour following 1.0% of HEC, HECVS low, HECVS medium and HECVS high at 24 hours. Data show the mean ±SE (n = 3). *Statistically significant according to t-test; $p < 0.05$ 157

The results of the tested materials from the fluorescent assay were as shown in Figure 6.4(a). It shows that HECVS has similar results despite the molar ratio of DVS to HEC and is significantly more irritating compared to HEC. Upon comparison with positive control 1.0% (w/v) BAC (a common ingredient in oral mouthwash products) for 1 hour as shown in Figure

6.4(b), exposure to 1.0% (w/v) of BAC results in a statistically significant increase in fluorescence intensity and causes 100% mortality after 1 hour exposure.

6.3.5. *In vitro* toxicity

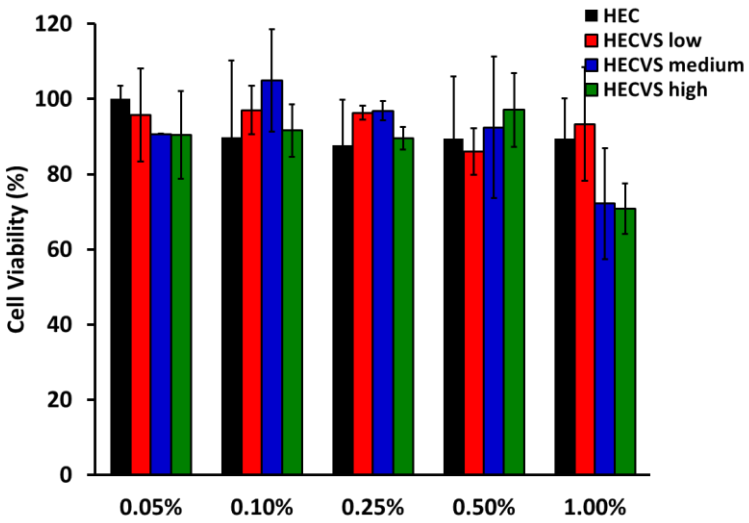


Figure 6.5. Caco-2 cell viability evaluated using MTT assay. Histograms represent the percentage of viable cells after the exposure to: 0.05%, 0.01%, 0.025%, 0.05% and 1.00% (w/v) HEC, HECVS low, HECVS medium and HECVS high. Data show the mean ±SE (n = 3). *Statistically significant according to t-test; $p < 0.05$

Figure 6.5 presents the cell viability results after exposure to 0.05%, 0.01%, 0.025%, 0.05% and 1.00% (w/v) of the new modified polymer HECVS. The results showed that both HEC and HECVS were not toxic to Caco-2 cells. In a cell culture study, DVS was reported to be a potent molecule as it showed about 5- to 6-fold higher toxicity than ethyl vinyl sulfone in human colon cancer cells with an IC50 of 34 ± 3 μM [30]. DVS exhibited the highest cytotoxicity with LC50 values of about 14–15 μM in HepG2 cells [31]. Although the planarian test in this study showed signs of toxicity, the modified polymer was found to be toxicologically safe in the *in vitro* cell viability test with percent of viability above 60%.,

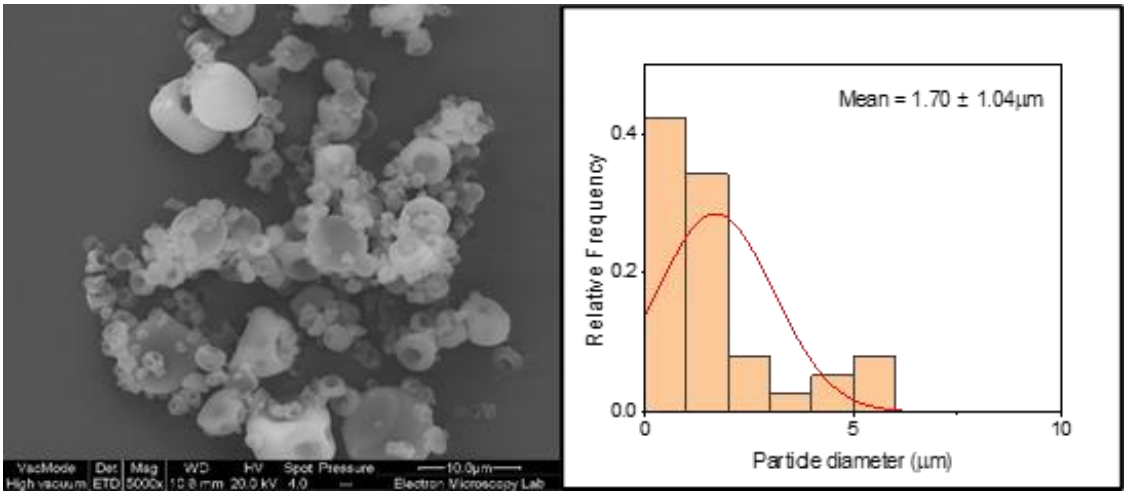
6.3.6. Physical characterisation of HECVS microparticles

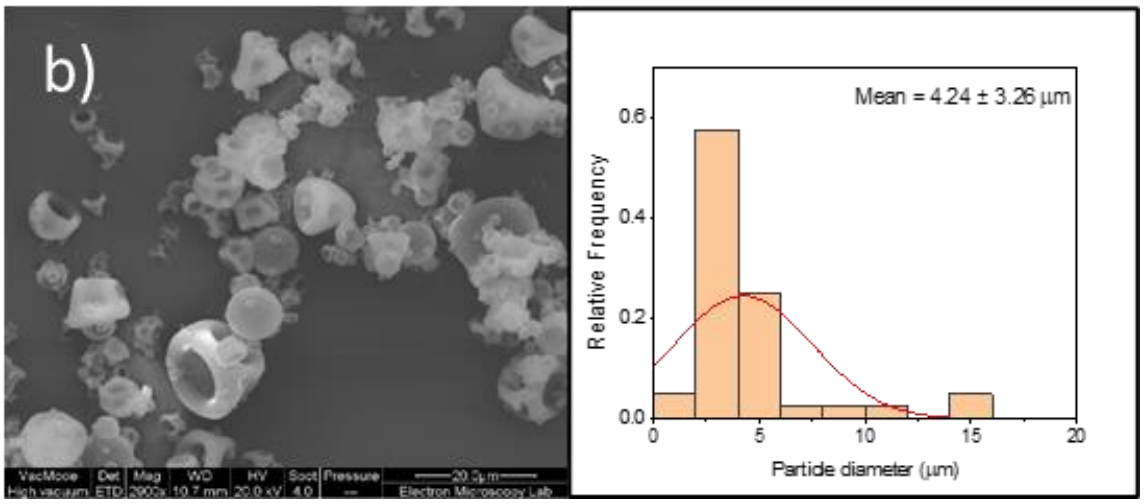
After spray drying the solutions, the morphology of the particles was determined by scanning electron microscopy (SEM) and the size was measured using ImageJ software (Figure 6.6). The particles are polydisperse in size with a spherical, collapsed shape. Several publications report that the possible reason for the collapsed shape (buckled shells) is the rapid drying

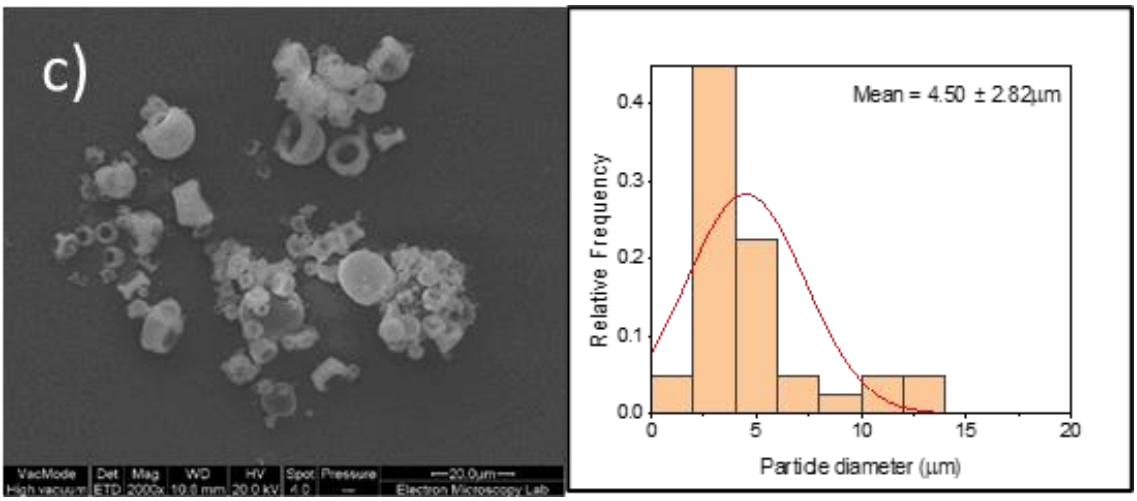
process [26,27]. The term "polydispersity" refers to the distribution of particle sizes within a sample, where the size of the particles varies. The polydispersity of the particles was attributed to the viscosity of the solutions, which led to the inhomogeneous drying of the solutions [26].

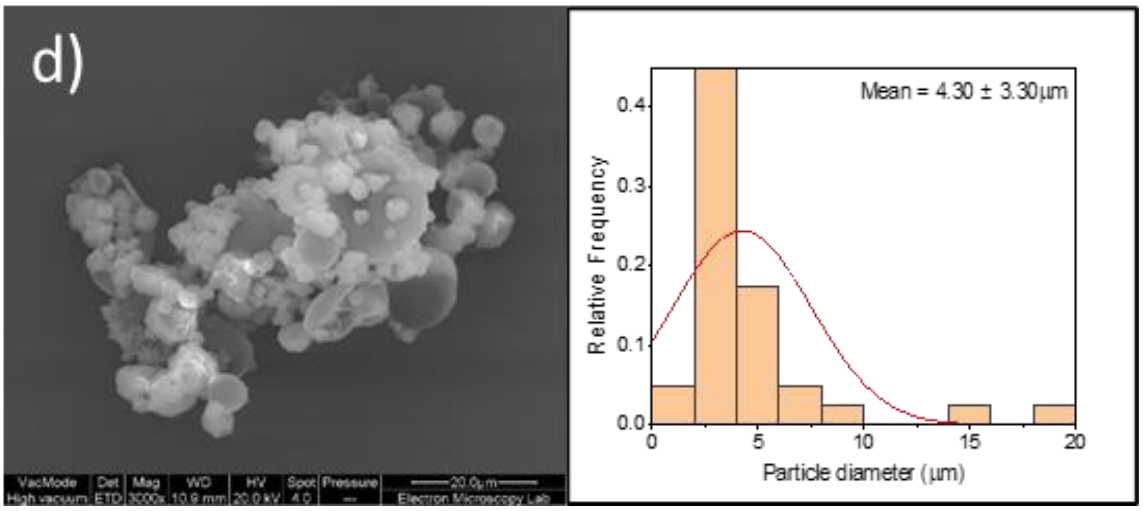
Based on the images from SEM, we have found that the size of the spray-dried particles for all polymers ranged from 1.70 ± 1.04 – 6.90 ± 3.56 µm. For the control solution chitosan, we have found that the size of the particles increased with increasing molecular weight of the chitosan. This is consistent with the reports by Sun et al., 2009. in which they found that the viscosity of the solution increases with increasing molecular weight, resulting in larger droplets being sprayed [28].

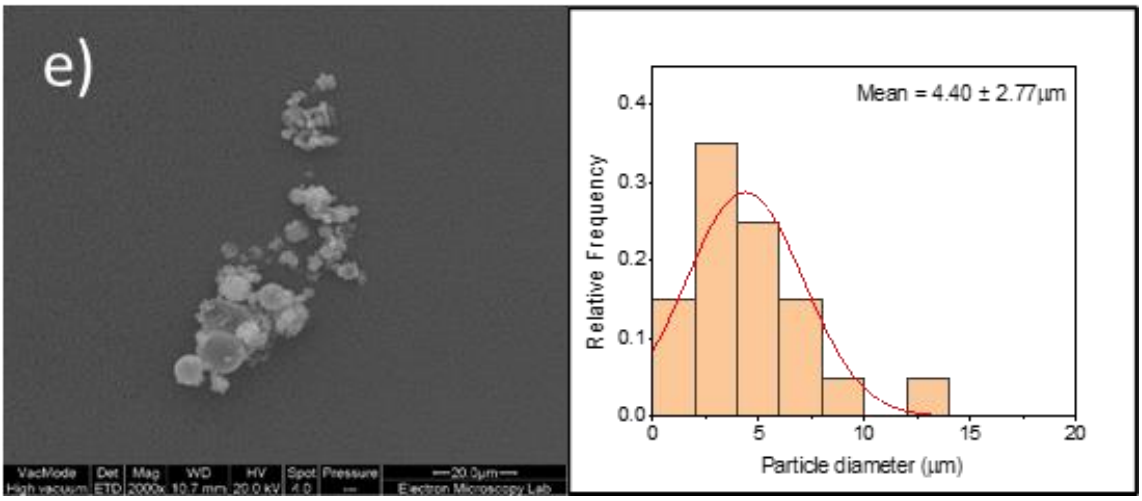
For modified HECVS, we saw the same trend as the higher the molar ratio, the larger the particles. Unmodified HEC has a smaller particle size (mean 1.70 ± 1.04 µm) compared to HECVS (mean 4.40 ± 2.77 µm – 5.58 ±2.40 µm). This indicates that the amount of conjugated DVS influences the size of the particles.






c)
Mean = 4.50 ± 2.82µm
Relative Frequency
Particle diameter (µm)


d)
Mean = 4.30 ± 3.30µm
Relative Frequency
Particle diameter (µm)


e)
Mean = 4.40 ± 2.77µm
Relative Frequency
Particle diameter (µm)

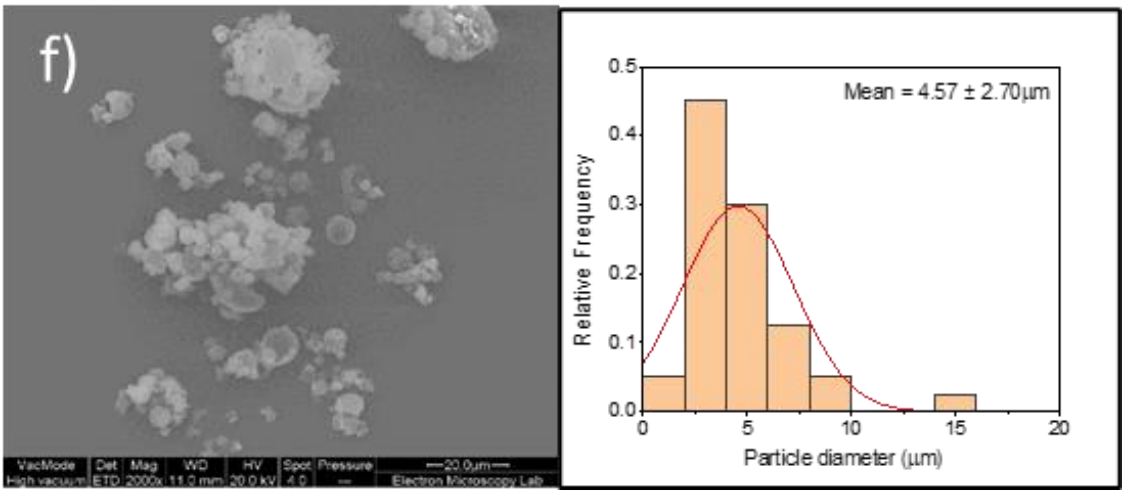
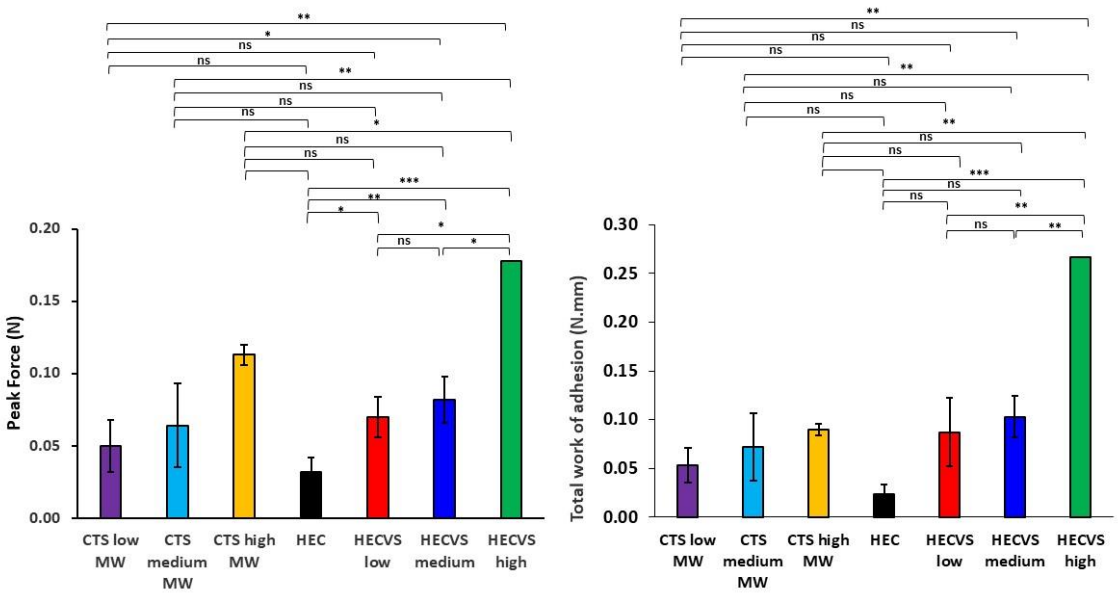
€(e 

Figure 6.6. Physical morphology of microparticles at various concentrations from (a) Chitosan low MW (b) Chitosan medium MW (c) Chitosan high MW (d) HEC (e) HECVS low (f) HECVS medium and (g) HECVS high

6.3.7. *Ex vivo* mucoadhesion profile of HECVS microparticles



(a) (b)

Figure 6.7. The mucoadhesion profile of unmodified HEC, chitosan low MW, chitosan medium MW, chitosan high MW, HECVS low, HECVS medium and HECVS high (a) Maximum peak force (N) and (b) Total work of adhesion (mm.N)

The maximum peak force is the highest height of the peak where it represents the maximum force (Fmax) needed to detach the probe away from the tissue. While work of adhesion is work or energy that is needed to separate two adhering surfaces. Accordingly, both the values for peak force and total work of adhesion appear to be similar and valid to rank the mucoadhesive properties of the polymers.

Figure 6.7 shows that the HECVS high has the greatest maximum peak force of 0.18 ± 0.02 N and a total work of adhesion of 0.27 ± 0.02 N.mm. The improvement in mucoadhesion of HECVS high was followed by HECVS medium and HECVS low under the same conditions. The results of these studies are consistent with the improved mucoadhesive properties of HEC modified with methacryloyl and maleimide groups in our previous studies [25,29]. This can be explained by a determining factor, namely the vinyl sulfone as a functional group with a strong electron deficiency, which is suitable for thiol-Michael addition reactions [30].

Interestingly, in this work we found that HECVS high has higher mucoadhesive properties than chitosan high MW as a positive control. Chitosan are cationic polymers and have good mucoadhesive properties due to a strong electrostatic interaction between the positively charged amines of chitosan and the negatively charged sialic acid residues of mucin. However,

oral administration of products containing chitosan is not very popular because chitosan precipitates at a higher pH (6-6.5), which may contribute to the low mucoadhesion compared to HECVS high in this study [31].

6.4. Discussion

Vinyl sulfone acts as an acceptor for the Michael reaction and reacts preferentially with nucleophiles such as mercapto, amines and thiols [32,33]. The reaction of vinyl sulfone with mercapto groups occurs in neutral buffers, but the reactions of vinyl sulfone with amino and hydroxyl groups typically occur in basic solutions [7,13,17,33].

In this work, HEC was functionalised with divinyl sulfone moieties in one pot with two steps chemical synthesis reaction. First, the hydroxyl group HEC were reacted with mercaptoproponic acid (an organosulfur compound with a bifunctional molecule, containing both carboxylic acid and thiol groups) using DMSO as a solvent to form vinyl sulfone propionic acid. Using N, N-dicyclohexylcarbodiimide (DCC)/4(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) as a catalyst system, HEC is conjugated with vinyl sulfone propionic acid. The final product is HEC-conjugated vinyl sulfone propionic acid by a substitution reaction between a carboxylic acid (R−C(=O)−OH) and alcohol (R'OH), forming an ester with an ethyl spacer between the thioether and ester groups (refer to a chemical structure in Figure 6.1).

We successfully synthesised the HECVS polymers, which was confirmed by the presence of the vinyl sulfone group using ^{1}H NMR and FTIR spectra. For further studies, the newly synthesised HECVS was successfully spray-dried with mean values ranging from 4.40 ± 2.77 µm to 5.58 ±2.40 µm. We used microparticles as a model dosage form to evaluate the mucoadhesion properties of HECVS in combination with chitosan of different molecular weights as a positive control.

The results of the mucoadhesion study showed that HECVS significantly improved the mucoadhesive properties of HEC. It was previously found that the more electron-deficient the alkene, the higher the kinetic rate of Michael addition of thiols [30]. In this study, modified HECVS was more reactive compared to the positive control group. This was confirmed by some studies showing that divinyl sulfone is very reactive, followed by acrylate and fumarate [30,34]. In addition to the thiol-Michael addition reaction, other interactions that form weak bonds such as van der Waals bonds or hydrogen bonds can also contribute to adhesion to the mucosal surface [35].

Despite the benefits it offers, divinyl sulfone is known to be a hazardous substance, especially when it comes into contact with the skin [29]. In the planarians acute toxicity test, results have shown that the safe concentration for HECVS high is below 0.25% (w/v), HECVS medium is below 0.5% (w/v) and HECVS low is below 1% (w/v). All newly modified HECVS were also found to be slightly more irritated than HEC from planaria fluorescent assay. This result, however, does not correlate with *in vitro* cytotoxicity tests showing viability at over 60% for all polymers.

6.5. Conclusion

The preliminary mucoadhesion profile from this work shows modified HECVS polymers are a new material for mucoadhesive drug delivery systems with improved mucoadhesive properties. However, the newly synthesised HECVS has been tested *in vitro* as safe for oral use in humans and potentially for other mucosal sites, such as the vaginal or ocular surface. This is contradicted by both the planaria acute toxicity test and the fluorescence test, which shows that all modified HECVS are potentially irritant and toxic if a high molar ratio of DVS to HEC modified HECVS was used. So further toxicological studies need to be carried out to support this.

6.6. References

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Chapter 7.

Concluding remarks and future work.

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To improve the mucoadhesive properties of non-ionic HEC, one of the strategies that has been proposed in the literature is to modify HEC with any unsaturated groups. These modified polymers can covalently bind to electronegative neighbouring groups such as sulfhydryl-containing biomolecules like mucin glycoprotein [1]. Recent studies successfully modified various types of polymers with acryloyl [2], methacryloyl [3,4] and maleimide groups [5,6]. However, modification of non-ionic polymers such as HEC with the unsaturated groups and modification of polymers with DVS (an unsaturated group) for mucoadhesion has not yet been reported. Modification with DVS tends to result in cross-linking and gel formation and thus requires an optimisation study [9].

All four objectives of this thesis were successfully achieved through Chapters 3-6, which covered works to modify hydroxyethyl cellulose with acryloyl, methacyloyl, maleimide and sulfone. As a new multifunctional excipient, this newly modified HEC with unsaturated groups has the advantage of preserving the non-ionic nature of the HEC derivatives, which may lead to better compatibility with charged drug molecules.

In the third chapter, a study of methacryloyl HEC as mucoadhesive wafers for buccal drug delivery was discussed. The modified HEC with methacryloyl group (HECGMA) was developed into wafers and the mucoadhesion study revealed that the highest molar ratio of the modified HECGMA exhibited the best mucoadhesive properties compared to other HECGMA modified polymers and the control HEC. The final product of modified HECGMA was found to be safe for human use based on both safety studies. It is therefore suggested that HECGMA with a high molar ratio to HEC is the best mucoadhesive excipient for this work. The new work in this chapter involves the use of GMA as a chemical for synthesis of the methacryloylated HEC. GMA can be chemically modified in two different pathways, either via transesterification or via epoxide ring opening mechanisms. We performed the synthesis in an alkaline protic solvent (with NaOH and TEA as bases), which resulted in the reaction favouring epoxide ring opening over transesterification. In a manufacturing set-up, it is best to simplify the synthesis process. One option is to perform the synthesis in an acidic environment, as GMA reacts with the hydroxyl groups of the macromolecules in an acidic aqueous solution only via an epoxide ring-opening mechanism.

The fourth chapter presents the synthesis and characterisation of HEC functionalised with maleimide groups. In this work, HECMAL solutions were sprayed onto blank tablets as a

dosage form to investigate the mucoadhesiveness of modified HECMAL polymers. The mucoadhesion study showed that the modified HECMAL with the highest molar ratio had the best mucoadhesive properties compared to the other modified HECMAL polymers and also to the control HEC. Toxicity studies showed that all modified polymers were safe. Thus, the modified HECMAL high is the best mucoadhesive excipient in this study and is recommended for further use. In this chapter, the chemical N-(4-bromophenyl) maleimide was used for modification, which was first reported in the conjugation of maleimide with polymers. There are a few points worth mentioning regarding HECMAL synthesis method. The chemical N-(4-bromophenyl) maleimide dissolved in an organic solvent and requires catalysis with TEA (which provides basic conditions) at low temperatures to better control the reaction and reduce the heat of the mild exothermic reaction.

The fifth chapter of this thesis focused on the synthesis of acryloylated HEC and the development of a mucoadhesive film for buccal delivery. The modified acryloylated HEC (HECAC) polymers use acryloyl chloride as the chemical for synthesis. In this work, a simple rheological study was conducted to investigate the interaction between HECAC and mucin. All mixtures of polymers and mucin show positive synergism. The highest viscosity ranking is with 1% (w/v) unmodified HEC + mucin, followed by 1% (w/v) HECAC medium + mucin, 1% (w/v) HECAC high + mucin and 1% (w/v) HECAC low + mucin. A similar pattern was observed when investigating mucoadhesion using a tensile test, where the modified HECAC medium has the best mucoadhesion properties compared to other modified HECAC and unmodified HEC polymers. The rheology experiments in this study require additional data to conclude the positive synergism and the determination of the force of bioadhesion value between polymers and mucin. The limitation of this work is that the study of interaction was only performed with 1% (w/v) polymer and 1% (w/v) mucin. Thus, variation of concentration is suggested for further study.

The sixth chapter covers the work on the synthesis of sulfonated HEC with divinyl sulfone. Divinyl sulfone is a highly toxic chemical that requires extra caution upon handling. The dosage form used to model the mucoadhesive properties of HECVS are microparticles prepared by spray drying modified HECVS solutions. The modified HECVS polymer has the highest mucoadhesive properties with modified HECVS at a high molar ratio. However, among all four modification studies, HECVS high and HECVS medium showed mild to strong toxicity signs via planaria acute toxicity assays and fluorescent assays. Therefore, it was proposed to modify HEC at a molar ratio of DVS less than 0.3 and at a working concentration below 0.5%

to ensure safe use. Further toxicological tests should be carried out using different *in vivo* and *in vitro* methods and models to confirm these preliminary data.

Unfortunately, synthesis with a low molar ratio of DVS to HEC results in a low DS. Modification with a high molar ratio leads to cross-linking between DVS and HEC and gel formation, as well as toxicity issues. Therefore, it is important to tune and optimise the stoichiometry, as the reaction can be saturated or stopped before cross-linking occurs, leaving unreacted vinyl sulfone groups for subsequent reactions.

Throughout this thesis, we showed methacryloyl, maleimide, acryloyl, and divinyl sulfone groups can all be considered highly reactive for Michael addition reactions with cysteine in the buccal region. Unfortunately, we are unable to rank the reactivity for Michael addition reaction in this thesis due to different dosage forms and this is not part of the objective. However, based on the high reactivity and electrophilic nature, Maleimide reacts very strongly with cysteine in Michael addition reactions. This is followed by other Michael acceptors: divinyl sulfone, acryloyl and methacryloyl groups [7]. For a convenient comparison, Table 7.1 provides summary of the methods and results from chapters 3-6 of this thesis.

The synthesis methods for HEC modification in this study were designed to be simple and have several advantages, such as scalability for larger production and a simple one-pot reaction. This reaction can proceed efficiently under mild conditions, so it can be carried out by non-chemists. However, there are some disadvantages. For example, the reaction can be challenging from a steric hindrance perspective, as the reaction often proceeds with low DS. Another limitation of this study is that no work was done on gel permeation chromatography (GPC) on polymers because the equipment was broken, and the outbreak of COVID-19 prolonged the repair process. GPC is mainly used to measure the actual molecular weight of the polymer compounds. Therefore, the molecular weight was calculated manually and according to the supplier's information.

In this thesis, several methods of quantification were used including integration of peaks in ^{1}H NMR, elemental analysis and HPLC to determine the extent of the substitution of functional groups and their distribution after modification of the polymers. However, not one method of quantification fits all modified HECs. Each method has its advantages and disadvantages.

For example, ^{1}H NMR spectroscopy is a very efficient, reliable, and common technique for determining the DS of polysaccharides. However, in very viscous environments with potential for aggregation processes and strong interactions between polymer chains, the accuracy of DS determination by traditional ^{1}H NMR is severely constrained [8]. Due to the complex structure

Table 7.1: Summary of methods and results from chapters 3-6 in the thesis

	Chapter 3 (HECGMA)	Chapter 4 (HECMAL)	Chapter 5 (HECAC)	Chapter 6 (HECVS)
Experimental methods				
Functional group	Methacryloyl	Maleimide	Acryloyl	Vinyl sulfone
Synthesis: chemical	Glycidyl methacrylate	N-(4-bromophenyl) maleimide	Acryloyl chloride	Divinyl sulfone
Synthesis: solvent	0.1 M NaOH	50% DMF + 50% DIW	Trifluoroacetic acid	DMSO
Synthesis: group	• HECGMA Low - molar ratio [HEC]:[GMA] of [1]:[1] • HECGMA Medium - molar ratio [HEC]:[GMA] of [1]:[2] • HECGMA High - molar ratio [HEC]:[GMA] of [1]:[3]	• HECMAL Low - molar ratio [HEC]:[BPM] of [1]:[1] • HECMAL Medium - molar ratio [HEC]:[BPM] of [1]:[2] • HECMAL High - molar ratio [HEC]:[BPM] of [1]:[3]	• HECAC Low - molar ratio [HEC]:[AC] of [1]:[1] • HECAC Medium - molar ratio [HEC]:[AC] of [1]:[2] • HECAC High - molar ratio [HEC]:[AC] of [1]:[3]	• HECVS Low - molar ratio [HEC]:[DVS] of [1]:[0.1] • HECVS Medum - molar ratio [HEC]:[DVS] of [1]:[0.3] • HECAC High - molar ratio [HEC]:[AC] of [1]:[0.9]
Dosage form	Thick wafer	Spray coated tablet	Film	Microparticles
Chemical characterization	• ^{1}H NMR • FTIR	• ^{1}H NMR • FTIR	• ^{1}H NMR • FTIR	• ^{1}H NMR • FTIR
Physical characterization of dosage form	• Size, weight & colour • Surface morphology – SEM images	• Size, weight & colour • Thickness of coated tablet – Fluorescent images	• Size, weight & colour • Thickness of film – SEM images	• Colour • Shape & size – SEM images
Quantification methods	• HPLC	• ^{1}H NMR • Elemental analysis	• HPLC	• ^{1}H NMR • Elemental analysis

Toxicity	• *In vivo* – Planaria Acute toxicity & fluorescent test • *In vitro* – Caco-2 cell			• *In vivo* – *In vivo* – Planaria Acute toxicity & fluorescent test • *In vitro* – Caco-2 cell			• *In vivo* – *In vivo* – Planaria Acute toxicity & fluorescent test • *In vitro* – Caco-2 cell			• *In vivo* – *In vivo* – Planaria Acute toxicity & fluorescent test • *In vitro* – Caco-2 cell		
Mucoadhesion methods/instrument	• Texture analyser – tensile test			• Texture analyser – tensile test			• Texture analyser – tensile test • Rheology – mucin + polymer synergism test			• Texture analyser – tensile test		
Results												
	HECGMA Low	HECGMA Medium	HECGMA High	HECMAL Low	HECMAL Medium	HECMAL High	HECAC Low	HECAC Medium	HECAC High	HECVS Low	HECVS Medium	HECVS High
DS: ^{1}H NMR				0.08	0.13	0.23						
Amount (µmol/gram): HPLC	64.49 ± 5.98	72.43 ± 6.16	173.50 ± 32.84				191.06 ± -7.31	273.78 ± 6.21	379.38± 56.14			
DS: Elemental analysis				0.07 ± 0.01	0.11 ± 0.00	0.22 ± 0.03				0.09 ± 0.01	1.59 ± 0.01	0.12 ± 0.00
MPF: Tensile test (N)	0.23 ± 0.04	0.54 ± 0.08	0.54 ± 0.09	0.04 ± 0.01	0.05 ± 0.003	0.06 ± 0.01	0.40 ± 0.02	0.46 ± 0.02	0.36 ± 0.05	0.07 ± 0.01	0.08 ± 0.01	0.18 ± 0.02
TWA: Tensile test (mm.N)	1.39 ± 0.02	1.46 ± 0.09	3.19 ± 0.09	0.05 ± 0.00	0.07 ± 0.01	0.11 ± 0.02	0.54 ± 0.06	1.06 ± 0.13	0.64 ± 0.20	0.09 ± 0.01	0.10 ± 0.04	0.27 ± 0.02

of the final synthesised products, only DS of HECMAL polymers was determined using this method.

For HECMAL and HECVS polymers, an elemental analysis method was used to determine the elementary constituents and further calculate the quantity of the functional group. The percentages of carbon, hydrogen, sulfur, and nitrogen in a sample can be accurately and precisely determined using this effective and simple procedure. It is also a commonly accepted technique in chemistry. The sample sent for the analysis must be dried before testing because moisture influences the quantitative outcome of the analysis.

HPLC have a high advantage as it can detect a wide range of analytes, from ions and tiny organic molecules to big macromolecules and polymers [9]. Another advantage is data can be replicated and are highly repeatable with a high level of confidence. However, the development and validation of methods using HPLC are time-bound events and thus require careful planning to develop a robust procedure. The HPLC method used in this thesis provides an efficient way of quantifying samples that are dissolved in water.

Other options to quantify functional moieties is by using a cheap, simple and rapid *in vitro* spectrophotometric assay such as inversed/indirect Elman's assay (detection of unreacted cysteine corresponding to functional moieties conjugated to the polymer) [10]. Another approach to determine the amount of DS is the detection of carbon double bonds with the alkaline potassium permanganate test (Baeyer test) [11]. Unfortunately, the results of these tests are not good and repeatable for all four modified HECs. Therefore, one cannot generally rely on a single analytical technique to quantify a sample. A second technique is valuable to confirm the accuracy of the primary technique;.

It is suggested that future research investigate the physical properties of the modified polymer for further use. This will be done through in-depth characterisation of the thermal, mechanical and structural properties of the modified polymers. This will provide a better knowledge of the structure–function relationships for new applications of the polymers. Therefore, the characterisation of the thermal properties using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) is proposed. The nanostructure of the polymer can be studied using small angle X-ray scattering (SAXS).

Future work should also address the theory of mucoadhesive bonding by Michael addition reaction by performing experiments using ^{1}H NMR and rheological study. The ^{1}H NMR spectra can provide data on the interactions of the molecular moieties of the mucin and polymer components based on their chemical shifts, and the mobility of each segment is determined by its line width following method by Pham et al [12]. We can distinguish whether the polymers

interact with specific segments of mucin or they have a universal effect on the mobility of all molecular segments of mucin. According to the changes in the mucin's ^{1}H signals, the interactions between the mucin and the polymers can be categorised into one of the following groups: I) When the relative changes of the N-acetyl (–$NHCOCH_3$) groups are smaller than their standard deviations, the ^{1}H signals of the mucin do not change; ii) ^{1}H signals are broadened but still detectable, known as the weak broadening effect; and iii) when they are broadened beyond the detection limit and do not produce any observable signal, known as the strong broadening effect. The advantage of this method is the mucin-polymer interactions can be determined without having to separate the free mucin and free polymer from the mucin-polymer complex in their mixes. This approach added information about the molecular interactions between mucin and the mucoadhesive polymer, which were not established in the thesis.

The rheological study of mucoadhesive/mucilage interaction of modified polymers allows measurement of the strength of mucoadhesive interaction, which is called rheological synergism. The polymer-mucilage interaction can be investigated using dynamic oscillatory rheology, which was developed by Mortazavi et al [13]. Briefly, mucus was mixed with polymer solution and the dynamic viscosity of each sample was evaluated by measuring in the shear rate range of 0.01–50.0 Pa at a frequency of 1 Hz. The increase in viscosity of modified polymer with mucus characterizes the mucoadhesive properties of the polymer, namely the greater the viscosity, the greater the mucoadhesive properties. Although it was demonstrated in the thesis, the bioadhesion force of the system was not quantitatively calculated. As previously mentioned in Chapter 5, variation of concentration is advised for future research to study the interaction between mucin and mucoadhesive polymer.

Lastly, this study only provides a preliminary proof of concept and requires additional data to comply with the regulatory requirement for registration of a new excipient. Registration of new excipients requires 2-5 years to complete the data collection before approval. Thus, this work provides a great start for the next step to meet the regulatory requirements such as more comprehensive toxicity data and preclinical trials with drugs loaded.

7.1. References

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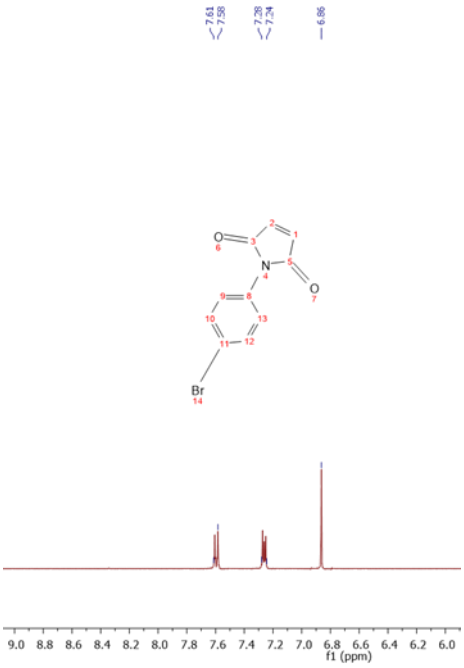
Appendix I

Standard calibration data of methacrylic acid

No	ID	Concentration (µmol/mL)	Peak area (DAD @ 200 nm)
1	MA 1	59.0	6672.8
2	MA 2	29.5	3462.7
3	MA 3	14.7	1765.2
4	MA 4	7.4	881.8
5	MA 5	3.7	352.9
6	MA 6	1.8	166.8
7	MA 7	0.9	99.9
8	MA 8	0.5	50.2
9	MA 9	0.2	24.6
10	MA 10	0.1	17.3

Appendix II



^{1}H NMR spectra of 4-N-BPM recorded in DMSO-d6

Appendix III

Elemental analysis results for HECMAL polymers

ELEMENT	**C**	**H**	**N**
$HECMAL_{low}$			
% Replicate 1	45.92	6.78	0.36
% Replicate 2	45.82	6.90	0.43
AVG	45.87	6.84	0.40
SD	0.07	0.08	0.05
$HECMAL_{medium}$			
% Replicate 1	46.15	6.51	0.61
% Replicate 2	46.07	6.71	0.62
AVG	46.11	6.61	0.62
SD	0.06	0.14	0.01
$HECMAL_{high}$			
% Replicate 1	46.55	5.93	1.35
% Replicate 2	46.35	6.13	1.08
AVG	46.45	6.03	1.22
SD	0.14	0.14	0.19

Appendix IV

Calculation of DS of amino moieties in HECMAL polymers

$$\frac{M_{AGU}.\,N\%}{MN.100-M_{SG}.N\%} \qquad (2)$$

Sample	N%	238*N%	(1400)-(76*N%)	$\frac{238*N\%}{(1400)-(76*N\%)}$
$HECMAL_{low}$	0.40 ± 0.05	94.01	1369.98	0.07 ± 0.01
$HECMAL_{medium}$	0.62 ± 0.01	146.37	1353.26	0.11 ± 0.00
$HECMAL_{high}$	1.22 ± 0.19	289.17	1307.66	0.22 ± 0.03

Appendix V

^{1}H NMR integration results for HECMAL polymers

	$HECMAL_{low}$	$HECMAL_{medium}$	$HECMAL_{high}$
IH maleimide	0.06	0.12	0.2
IH HEC	569.78	755.27	693.95

Appendix VI

Calculation of malemide from ^{1}H NMR integration results

$$DS = \frac{\text{IH maleimide}/2}{\text{IH HEC}/16+\text{IH maleimide}/2} \quad (1)$$

Sample	IH maleimide/2	IH HEC/16	IH HEC/16 + IH maleimide/2	% $\frac{\text{IH maleimide}/2}{\text{IH HEC}/16 + \text{IH maleimide}/2}$
$HECMAL_{low}$	0.03	35.61	35.64	0.08
$HECMAL_{medium}$	0.06	47.20	47.26	0.13
$HECMAL_{high}$	0.10	43.37	43.47	0.23

Appendix VII

Standard calibration data of acrylic acid

No	ID	Concentration (μmol/mL)	Peak area
1	AA 1	72.9	12603.3
2	AA 2	36.5	6031.1
3	AA 3	18.2	2992.9
4	AA 4	9.1	1490.9
5	AA 5	4.6	740.3
6	AA 6	2.3	366.2
7	AA 7	1.1	181.1
8	AA 8	0.6	89.1
9	AA 9	0.3	43.4
10	AA 10	0.1	18.8

Appendix VIII

Elemental analysis results for HECVS polymers

ELEMENT	C	H	N	S
HECVS low				
% Replicate 1	45.37	6.87	0.41	1.06
% Replicate 2	45.61	7.19	0.34	1.01
AVG	45.49	7.03	0.38	1.04
SD	0.17	0.23	0.05	0.04
HECVS medium				
% Replicate 1	43.89	7.16	0.77	1.06
% Replicate 2	46.37	7.38	0.53	1.36
AVG	45.13	7.27	0.65	1.21
SD	1.75	0.16	0.17	0.21
HECVS high				
% Replicate 1	43.50	6.87	0.69	1.58
% Replicate 2	46.59	7.16	0.37	1.59
AVG	45.05	7.02	0.53	1.59
SD	2.18	0.21	0.23	0.01

Appendix IX

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Calculation of DS of sulfate moieties in modified HECVS polymers

$$\frac{228.N\%}{3200-102.N\%} \quad (1)$$

Sample	S%	228*N%	3200-(102*N%)	$\frac{228*N\%}{3200-(102*N\%)}$
HECVS low	1.04 ± 0.04	241.68	3091.88	0.08 ± 0.00
HECVS medium	1.21 ± 0.21	275.88	3076.58	0.09 ± 0.01
HECVS high	1.59 ± 0.01	362.52	3037.82	0.12 ± 0.00